WEST

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L1: Entry 1 of 1 File: DWPI Mar 15, 1985

DERWENT-ACC-NO: 1985-102448
DERWENT-WEEK: 198517

TITLE: Determn. of antigen or antibody in blood - by adding haemolytic agent and antigen-or antibody-sensitised carrier suspension to blood sample and observing haemagglutination

PATENT-ASSIGNEE:

ASSIGNEE	CODE
TERUMO CORP	TERU

PRIORITY-DATA: 1983JP-0154922 (August 26, 1983)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
JP 60047962 A	March 15, 1985		003	
JP 90051150 B	November 6, 1990		000	

APPLICATION-DATA:

PUB-NO	APPL-DATE	APPL-NO	DESCRIPTOR
JP 60047962A	August 26, 1983	1983JP-0154922	
JP 90051150B	August 26, 1983	1983JP-0154922	

INT-CL (IPC): A61K 39/44; G01N 33/54

ABSTRACTED-PUB-NO: JP 60047962A
BASIC-ABSTRACT:

Method comprises adding a haemolytic agent and a suspension of carrier sensitised with antigen or antibody to a blood sample and observing the resultant haemagglutination.

Examples of haemolytic agent are saponins and surfactants. Blood sample is dissolved in haemolytic agent before haemagglutination, or a haemolytic agent is added to antigen- or antibody-sensitised carrier suspension to a concn. of 0.2-2.0% and then blood sample is dissolved in the suspension. The carrier is latex resin, inorganic adsorbent or immobilised red blood cells.

USE/ADVANTAGE - Method is useful in diagnosis of chronic arthritic rheumatism. It is unnecessary to prepare serum from blood to be examined, since red blood cells do not interfere with observation of haemagglutination.

CHOSEN-DRAWING: Dwg.0/0

TITLE-TERMS: DETERMINE ANTIGEN ANTIBODY BLOOD ADD HAEMOLYTIC AGENT ANTIGEN ANTIBODY SENSITIVE CARRY SUSPENSION BLOOD SAMPLE OBSERVE HAEMAGGLUTINATION

DERWENT-CLASS: A96 B04

CPI-CODES: A12-V03C; A12-W11B; B04-A07E; B04-B04C; B04-B04D; B11-C07A; B12-D03; B12-D09; B12-K04; B12-M09;

CHEMICAL-CODES:

Chemical Indexing M1 *01*
Fragmentation Code
M423 M750 M903 N102 V600 V611 V641 V791

Chemical Indexing M1 *02*
Fragmentation Code
M423 M760 M903 N102 V600 V614 V615

Chemical Indexing M1 *03*
Fragmentation Code
M423 M430 M782 M903 N102 P831 V400 V402

Chemical Indexing M1 *04*
Fragmentation Code
G010 G100 H7 H715 H721 M210 M212 M240 M281 M320
M423 M430 M510 M520 M531 M540 M610 M782 M903 N102
P831 V600 V611 V743 V752 V791

Chemical Indexing M6 *05*
Fragmentation Code
M903 P423 P831 Q616 R319 R515 R520 R611 R621 R622
R627 R630 R631 R639

POLYMER-MULTIPUNCH-CODES-AND-KEY-SERIALS:
Key Serials: 0231 0304 2504 2573 2651 3288

Multipunch Codes: 014 04- 055 056 397 436 532 536 57& 575 592 593 645 688

SECONDARY-ACC-NO:
CPI Secondary Accession Numbers: C1985-044410
Non-CPI Secondary Accession Numbers: N1985-076705

⑲日本国特許庁（JP）　⑪特許出願公開

⑫公開特許公報（A）　昭60－47962

⑤1Int.Cl.⁴	識別記号	庁内整理番号	㊸公開　昭和60年(1985)3月15日
G 01 N 33/543		7906－2G	
A 61 K 39/44		7043－4C	

審査請求　未請求　発明の数 2　（全3頁）

㊹発明の名称　血中の抗原または抗体量の測定方法およびそれに使用する試験液

㉑特　願　昭58－154922
㉒出　願　昭58(1983)8月26日

㉒発明者　伊藤　良孝　調布市小島町2丁目55番1号　調布南コーポラス805
㉑出願人　テルモ株式会社　東京都渋谷区幡ヶ谷2丁目44番1号
㉔代理人　弁理士　西村　公佑

明細書

1．発明の名称

血中の抗原または抗体量の測定方法およびそれに使用する試験液

2．特許請求の範囲

（1）全血検体に溶血剤と抗原または抗体感作担体浮遊液とを加え、その凝集反応を追跡することを特徴とする血中の抗原または抗体量の測定方法。

（2）溶血剤と抗原または抗体感作担体とを含有することを特徴とする血中の抗原または抗体量の測定方法に使用する試験液。

（3）溶血剤がサポニンである特許請求の範囲第2項記載の試験液。

3．発明の詳細な説明

I．発明の背景

技術分野

本発明は、血中の抗原または抗体量の測定方法およびそれに使用する試験液に関するものである。

さらに詳しくは、本発明は、血中の抗原または抗体量を免疫反応に基づく凝集反応により測定する方法およびそれに使用する試験液に関するものである。

本発明は、慢性関節リウマチの診断等各種の免疫学的検査に利用される。

先行技術およびその問題点

従来、凝集反応により血液中の抗原または抗体量を測定する場合、検体中に赤血球が存在すると肉眼による凝集の判定が困難であることから検体として血清が用いられていた。しかし検体の個数が多い場合等は血清の調製に相当の手間と時間を要する。また血清の調製のため、本来検査に必要としない余分な量の血液を採取しなければならない。

II．発明の目的

そこで本発明は、血清を調製する手間を省き、全血から直接抗原または抗体量を測定することができる方法を提供することを目的とする。

さらに本発明は、上記の測定方法に使用される

試験液を提供することを目的とする。

かかる目的を達成するため、本発明は、全血検体に溶血剤の抗原または抗体感作担体浮遊液とを加え、その凝集反応を追跡することを特徴とする血中の抗原または抗体量の測定方法からなる。

さらに本発明は、溶血剤と抗原または抗体感作担体とを含有する上記測定方法に使用される試験液からなる。

さらに本発明は、溶血剤がサポニンである上記試験液からなる。

III．発明の具体的説明

本発明の方法は、採取した全血検体に溶血剤と抗原または抗体感作担体浮遊液とを加え、その凝集反応を追跡することによって実施される。

上記方法において溶血剤としてはサポニンや各種の界面活性剤が使用される。溶血剤は凝集反応に先立って予め全血に加え、赤血球を溶解してもよく、あるいは、抗原または抗体感作担体浮遊液に約0.2～2%の濃度で加えておき、凝集反応の際に赤血球を溶解させてもよい。抗原または抗体感作担体としては、ラテックス樹脂、無機吸着剤、薬品処理した固定赤血球等従来公知のものが特に限定なく使用されうる。

凝集反応の追跡は常法に従って行なわれる。即ち、全血1滴をスライドグラス上に滴下し、これに溶血剤および抗原または抗体感作担体浮遊液の1滴を加え木の棒でよく混和し、およそ20×25㎜ぐらいにひろげる。スライドグラスを両手にもち、1分間ゆり動かした後凝集の有無を肉眼で判定する。その際赤血球は溶解しているので凝集判定の妨げにならない。

次に実施例を示して本発明をさらに具体的に説明する。

実施例

（1）リューマチ因子（RF）検出用ヒトガンマーグロブリン感作ラテックスの作成

グリシン－塩化ナトリウム緩衝液（pH8.2）（以下GNBと略称する）にポリスチレンラテックス（粒径0.117 μ）を固形分2.0%となるように加えて懸濁させる。一方、GNBに対して透析したヒトガンマーグロブリンを10㎎/㎖となるようにGNBに溶かす。両液を体積比1：1で混合し、50℃で1時間加温した。得られた液をGNBで遠心洗浄（17,000rpm、10分間）し、これに牛血清アルブミン0.5%、サポニン0.4%を含むGNBを加えて0.4%感作ラテックス浮遊液を作成した。以上の条件では感作蛋白濃度は10～100 μgN/㎖、ラテックス粒子密度は4.53×10^{8}個／㎖となり、ラテックス粒子1個当たり75,000個のガンマーグロブリン分子が結合すると概算された。

（2）スライド凝集反応

上記（1）で得られた感作ラテックス1滴（約0.02～0.03㎖）および血液または血清1滴を反応用スライドグラス上でよく混ぜ合わせ、直径約2㎝程度にひろげて凝集反応を行なった。スライドグラスを前後にゆり動かしながら1分後に凝集の有無、程度を次の判定基準に従い判定した。結果を表1に示す。

陽性（＋）

液全体に凝集塊が極めて多く、凝集していることが肉眼ではっきり認められる。

陰性（－）

肉眼では全く凝集が認められない。

判定不能（？）

ラテックスの凝集が判然としない。

陽性コントロール

慢性関節リウマチ（RA）コントロール血清

（凝集力価180）

陰性コントロール

健常人血清

上記コントロール血清と健常人濃厚赤血球とをそれぞれ再構成し（ヘマトクリット値40%）、被検体とした。

表1

	感作ラテックス 被検体	サポニン添加 感作ラテックス	サポニン無添加 感作ラテックス
陰性 コントロール	健常人血清	−	−
	全血（抗凝固剤無）	−	?
	全血（ヘパリン血）	−	?
	全血（ACD血）	−	?
陽性 コントロール	RA血清	+	+
	全血（抗凝固剤無）	+	?
	全血（ヘパリン血）	+	?
	全血（ACD血）	+	?

表1から、サポニン添加感作ラテックス試験液においては、検体として全血を使用しても判定が明瞭であり特異性に優れていることが明らかである。

Ⅳ．発明の具体的作用効果

本発明によれば、全血から直接抗原または抗体量を測定することができる血中の抗原または抗体量測定方法が提供される。

本発明の方法においては前述した如く溶血剤が使用され、赤血球が溶解するので検体として全血を用いても凝集の有無判定は容易である。従って従来の測定法におけるように、凝集試験のために血清を調製する必要がなく、測定操作が簡略化される。

さらに本発明によれば、上記の測定法に好適に使用される試験液が提供される。本発明の試験液は、溶血剤と抗原または抗体感作担体とを含有しているので、これを全血と混合するだけで赤血球が溶け、凝集の判定が容易に行なわれる。

(FILE 'HOME' ENTERED AT 09:50:31 ON 30 JUL 2003)

FILE 'BIOSIS, CAPLUS, EMBASE, MEDLINE, CANCERLIT, JAPIO' ENTERED AT 09:50:48 ON 30 JUL 2003

L1	0	S	(LYSI? BEFORE AGGLUTINATION)
L2	0	S	(LYSI? PRIOR TO AGGLUTINATION)
L3	745	S	LYSIS AND AGGLUTINATION
L4	123	S	(LYSI? BEFORE)
L5	48		DUPLICATE REMOVE L4 (75 DUPLICATES REMOVED)
L6	1	S	L5 AND (WHOLE BLOOD)
L7	0	S	(LYSI? BEFORE AGGLUTINATION)
L8	192	S	L3 AND ERYTHROCYTE?
L9	96	S	L8 AND BLOOD?
L10	3	S	L9 AND PARTICLE?

=>

10 ANSWER 3 OF 3 CAPLUS COPYRIGHT 2003 ACS on STN
AN 1962:445467 CAPLUS
DN 57:45467
OREF 57:9085d-f
TI Immune **agglutination** and **lysis** of antigen-modified **erythrocytes** from various animal species
AU Bokkenheuser, V.; Gorzynski, E. A.; Cohen, E.; Neter, E.
CS Univ. of Buffalo, Buffalo, NY
SO Soc. Exptl. Biol. Med. (1962), 110, 94-8
DT Journal
LA Unavailable
CC 70 (Immunochemistry)
AB **Agglutination** and **lysis** by antibodies of **erythrocytes**, modified by pretreatment with corresponding antigens, were studied. **Erythrocytes** from man, sheep, and goat, modified by O antigens from Enterobacteriaceae and heterogenetic (staphylococcal) antigens, are readily agglutinated by the sp. antibodies and treatment with proteolytic enzymes only slightly enhances the **agglutination** reaction. Similarly treated **erythrocytes** from alligator and ox are not agglutinated, or agglutinated only in low titer; enzyme treatment markedly enhances the reaction. **Erythrocytes** from all these animal species modified by Vi antigen are readily agglutinated by Vi antiserum in high titer. Poorly agglutinable ox **erythrocytes** modified by O antigen are lysed in presence of O antibody and guinea pig complement. Vi antigen present together with O antigen on latex **particles** and **erythrocytes** significantly inhibits **agglutination** by O antibodies.
IT Hemagglutination
(after **erythrocyte** treatment with antigens, spp. and)
IT **Blood** corpuscles, red.
(antigen-treated, of different species)
IT Staphylococcus
(antigens O and Vi, **agglutination** and **lysis** of **erythrocytes** treated with)
IT Enterobacteriaceae
(antigens O of, **agglutination** and **lysis** of **erythrocytes** treated with)
IT Antigens
(**erythrocytes** treated with, hemagglutination and hemolysis of, spp. in relation to)
IT Antibodies
(to **erythrocytes**, after antigen treatment)
IT Hemolysis., adenosine triphosphate
(of antigen-modified **erythrocytes**, spp. and)

10 ANSWER 3 OF 3 CAPLUS COPYRIGHT 2003 ACS on STN
AN 1962:445467 CAPLUS
DN 57:45467
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TI Immune **agglutination** and **lysis** of antigen-modified **erythrocytes** from various animal species
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SO Soc. Exptl. Biol. Med. (1962), 110, 94-8
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IT Antibodies
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IT Hemolysis., adenosine triphosphate
(of antigen-modified **erythrocytes**, spp. and)

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L1	0	S	(LYSI? BEFORE AGGLUTINATION)
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L5	48		DUPLICATE REMOVE L4 (75 DUPLICATES REMOVED)
L6	1	S	L5 AND (WHOLE BLOOD)
L7	0	S	(LYSI? BEFORE AGGLUTINATION)
L8	192	S	L3 AND ERYTHROCYTE?
L9	96	S	L8 AND BLOOD?
L10	3	S	L9 AND PARTICLE?

=>

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L1: Entry 1 of 1 File: DWPI Mar 15, 1985

DERWENT-ACC-NO: 1985-102448
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PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
JP 60047962 A	March 15, 1985		003	
JP 90051150 B	November 6, 1990		000	

APPLICATION-DATA:

PUB-NO	APPL-DATE	APPL-NO	DESCRIPTOR
JP 60047962A	August 26, 1983	1983JP-0154922	
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INT-CL (IPC): A61K 39/44; G01N 33/54

ABSTRACTED-PUB-NO: JP 60047962A
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USE/ADVANTAGE - Method is useful in diagnosis of chronic arthritic rheumatism. It is unnecessary to prepare serum from blood to be examined, since red blood cells do not interfere with observation of haemagglutination.

CHOSEN-DRAWING: Dwg.0/0

TITLE-TERMS: DETERMINE ANTIGEN ANTIBODY BLOOD ADD HAEMOLYTIC AGENT ANTIGEN ANTIBODY SENSITIVE CARRY SUSPENSION BLOOD SAMPLE OBSERVE HAEMAGGLUTINATION

DERWENT-CLASS: A96 B04

CPI-CODES: A12-V03C; A12-W11B; B04-A07E; B04-B04C; B04-B04D; B11-C07A; B12-D03; B12-D09; B12-K04; B12-M09;

CHEMICAL-CODES:

Chemical Indexing M1 *01*
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M423 M750 M903 N102 V600 V611 V641 V791

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Chemical Indexing M1 *03*
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M423 M430 M782 M903 N102 P831 V400 V402

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G010 G100 H7 H715 H721 M210 M212 M240 M281 M320
M423 M430 M510 M520 M531 M540 M610 M782 M903 N102
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R627 R630 R631 R639

POLYMER-MULTIPUNCH-CODES-AND-KEY-SERIALS:
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SECONDARY-ACC-NO:
CPI Secondary Accession Numbers: C1985-044410
Non-CPI Secondary Accession Numbers: N1985-076705

7 ANSWER 1 OF 3 CAPLUS COPYRIGHT 2003 ACS on STN
AN 2002:87271 CAPLUS
DN 136:98839
TI **Whole blood** immunoassay
IN Uchida, Shinya; Konishi, Aya; Torii, Tsuneyoshi; Nakashima, Kazuhiro
PA Sysmex Corporation, Japan
SO Eur. Pat. Appl., 6 pp.
CODEN: EPXXDW
DT Patent
LA English
IC ICM G01N033-543
CC 9-10 (Biochemical Methods)
Section cross-reference(s): 10, 14
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI EP 1176424	A2	20020130	EP 2001-116744	20010719
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI, RO				
JP 2002107365	A2	20020410	JP 2001-206798	20010706
US 2002031791	A1	20020314	US 2001-915580	20010727
PRAI JP 2000-226270	A	20000727		

AB A **whole blood** immunoassay includes the steps of mixing a **whole blood** sample with sensitized insol. carrier **particles** to cause an immune **agglutination**; dilg. the resulting **agglutination** mixt. with an aq. soln. contg. an erythrocyte **lysing** agent to lyse erythrocytes, thereby prepg. an assay sample; and detg. a degree of **agglutination** of the assay sample.

ST **blood** immunoassay

IT Immunoassay
(**agglutination** test; **whole blood** immunoassay)

IT Immunoassay
(app.; **whole blood** immunoassay)

IT Cytometry
(flow; **whole blood** immunoassay)

IT Antigens
RL: ANT (Analyte); THU (Therapeutic use); ANST (Analytical study); BIOL (Biological study); USES (Uses)
(hepatitis B surface; **whole blood** immunoassay)

IT **Blood** analysis
Carriers
Cytolysis
Dilution
Erythrocyte
Immunoassay
Light scattering
Mathematical methods
Mixing
Mixtures
Particle size distribution
Particles
Reaction
Solutions
Surfactants
Temperature
Test kits
Time
(**whole blood** immunoassay)

IT 151-21-3, Dodecylsodium sulfate, analysis
RL: ARU (Analytical role, unclassified); ANST (Analytical study)

(**whole blood** immunoassay)

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USE/ADVANTAGE - Method is useful in diagnosis of chronic arthritic rheumatism. It is unnecessary to prepare serum from blood to be examined, since red blood cells do not interfere with observation of haemagglutination.

CHOSEN-DRAWING: Dwg.0/0

TITLE-TERMS: DETERMINE ANTIGEN ANTIBODY BLOOD ADD HAEMOLYTIC AGENT ANTIGEN ANTIBODY SENSITIVE CARRY SUSPENSION BLOOD SAMPLE OBSERVE HAEMAGGLUTINATION

DERWENT-CLASS: A96 B04

CPI-CODES: A12-V03C; A12-W11B; B04-A07E; B04-B04C; B04-B04D; B11-C07A; B12-D03; B12-D09; B12-K04; B12-M09;

CHEMICAL-CODES:

Chemical Indexing M1 *01*
Fragmentation Code
M423 M750 M903 N102 V600 V611 V641 V791

Chemical Indexing M1 *02*
Fragmentation Code
M423 M760 M903 N102 V600 V614 V615

Chemical Indexing M1 *03*
Fragmentation Code
M423 M430 M782 M903 N102 P831 V400 V402

Chemical Indexing M1 *04*
Fragmentation Code
G010 G100 H7 H715 H721 M210 M212 M240 M281 M320
M423 M430 M510 M520 M531 M540 M610 M782 M903 N102
P831 V600 V611 V743 V752 V791

Chemical Indexing M6 *05*
Fragmentation Code
M903 P423 P831 Q616 R319 R515 R520 R611 R621 R622
R627 R630 R631 R639

POLYMER-MULTIPUNCH-CODES-AND-KEY-SERIALS:
Key Serials: 0231 0304 2504 2573 2651 3288

Multipunch Codes: 014 04- 055 056 397 436 532 536 57& 575 592 593 645 688

SECONDARY-ACC-NO:
CPI Secondary Accession Numbers: C1985-044410
Non-CPI Secondary Accession Numbers: N1985-076705

WEST

End of Result Set

Generate Collection Print

L1: Entry 1 of 1 File: DWPI Mar 15, 1985

DERWENT-ACC-NO: 1985-102448
DERWENT-WEEK: 198517

TITLE: Determn. of antigen or antibody in blood - by adding haemolytic agent and antigen-or antibody-sensitised carrier suspension to blood sample and observing haemagglutination

PATENT-ASSIGNEE:

ASSIGNEE	CODE
TERUMO CORP	TERU

PRIORITY-DATA: 1983JP-0154922 (August 26, 1983)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
JP 60047962 A	March 15, 1985		003	
JP 90051150 B	November 6, 1990		000	

APPLICATION-DATA:

PUB-NO	APPL-DATE	APPL-NO	DESCRIPTOR
JP 60047962A	August 26, 1983	1983JP-0154922	
JP 90051150B	August 26, 1983	1983JP-0154922	

INT-CL (IPC): A61K 39/44; G01N 33/54

ABSTRACTED-PUB-NO: JP 60047962A
BASIC-ABSTRACT:

Method comprises adding a haemolytic agent and a suspension of carrier sensitised with antigen or antibody to a blood sample and observing the resultant haemagglutination.

Examples of haemolytic agent are saponins and surfactants. Blood sample is dissolved in haemolytic agent before haemagglutination, or a haemolytic agent is added to antigen- or antibody-sensitised carrier suspension to a concn. of 0.2-2.0% and then blood sample is dissolved in the suspension. The carrier is latex resin, inorganic adsorbent or immobilised red blood cells.

USE/ADVANTAGE - Method is useful in diagnosis of chronic arthritic rheumatism. It is unnecessary to prepare serum from blood to be examined, since red blood cells do not interfere with observation of haemagglutination.

CHOSEN-DRAWING: Dwg.0/0

TITLE-TERMS: DETERMINE ANTIGEN ANTIBODY BLOOD ADD HAEMOLYTIC AGENT ANTIGEN ANTIBODY SENSITIVE CARRY SUSPENSION BLOOD SAMPLE OBSERVE HAEMAGGLUTINATION

DERWENT-CLASS: A96 B04

CPI-CODES: A12-V03C; A12-W11B; B04-A07E; B04-B04C; B04-B04D; B11-C07A; B12-D03; B12-D09; B12-K04; B12-M09;

CHEMICAL-CODES:

Chemical Indexing M1 *01*
Fragmentation Code
M423 M750 M903 N102 V600 V611 V641 V791

Chemical Indexing M1 *02*
Fragmentation Code
M423 M760 M903 N102 V600 V614 V615

Chemical Indexing M1 *03*
Fragmentation Code
M423 M430 M782 M903 N102 P831 V400 V402

Chemical Indexing M1 *04*
Fragmentation Code
G010 G100 H7 H715 H721 M210 M212 M240 M281 M320
M423 M430 M510 M520 M531 M540 M610 M782 M903 N102
P831 V600 V611 V743 V752 V791

Chemical Indexing M6 *05*
Fragmentation Code
M903 P423 P831 Q616 R319 R515 R520 R611 R621 R622
R627 R630 R631 R639

POLYMER-MULTIPUNCH-CODES-AND-KEY-SERIALS:
Key Serials: 0231 0304 2504 2573 2651 3288

Multipunch Codes: 014 04- 055 056 397 436 532 536 57& 575 592 593 645 688

SECONDARY-ACC-NO:
CPI Secondary Accession Numbers: C1985-044410
Non-CPI Secondary Accession Numbers: N1985-076705

L24 ANSWER 4 OF 4 CAPLUS COPYRIGHT 2002 ACS
AN 1989:512016 CAPLUS
DN 111:112016
TI Process for the rapid and simple isolation of nucleic acids and other heat-agglomeration-resistant water-soluble nitrogen-containing organic compounds
IN Holmes, David S.
PA State University of New York, Research Foundation, USA
SO U.S., 6 pp.
CODEN: USXXAM
DT Patent
LA English
IC ICM C12N001-06
ICS C12N001-08; C07G017-00; C07K003-12
NCL 435259000
CC 9-9 (Biochemical Methods)
Section cross-reference(s): 10
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
US 4830969	A	19890516	US 1981-298064	19810831

AB A process for the sepn. from other cellular materials of heat-agglomeration-resistant water-sol. N contg. org. compds. (e.g., plasmids, RNAs, mitochondrial DNAs, viral DNAs, chloroplast DNAs, other episomal DNAs and certain proteins) comprises heating cellular materials in a soln. of **lysing agent** to lyse the desired cells and to agglomerate water-sol. N-contg. compds. such as certain chromosomal DNAs which are not resistant to agglomeration; centrifuging the resulting product to remove water-sol. agglomerated materials; sepg. the supernatant liq. and pptg. the water-sol. agglomeration-resistant org. compds. with a water-sol. precipitant. The process also includes sepg. the agglomeration-resistant water-sol. N-contg. compds. from each other by means of exclusion chromotog. Yeast cells were suspended in a lysing soln. (urea 7, NaCl 0.35 M, EDTA 1 mM, and pH 8.0 Tris buffer 0.01M), SDS was added to 1%, and the soln. was rapidly brought to a boil and boiled 1 min. The soln. was centrifuged at 12,000 .times. g for 5 min and the supernatant was removed and pptd. with isopropanol at -18.degree. for .gtoreq.30 min. The purified RNA was collected by centrifuging at 12,000 .times. g for 10 min at 4.degree..
ST nucleic acid isolation heat agglomeration; RNA yeast purifn heat agglomeration
IT Chloroplast
Mitochondria
Virus
(DNA of, isolation and purifn. of, heat agglomeration in)
IT Escherichia coli
(RNA and plasmids of, isolation and purifn. of, heat agglomeration in)
IT Liver, composition
Soybean
Yeast
(RNA of, isolation and purifn. of, heat agglomeration in)
IT Agglomeration
Centrifugation
Chelating agents
Chromatography, gel
Precipitation
Surfactants
(in isolation and purifn. of nucleic acids and other heat-agglomeration-resistant water-sol. nitrogen-contg. org. compds.)
IT Plasmid and Episome
Deoxyribonucleic acids
Nucleic acids
Peptides, preparation
Proteins, preparation
Ribonucleic acids
RL: ANST (Analytical study)
(isolation and purifn. of, heat agglomeration in)
IT Mouse
(liver cells of, RNA of, isolation and purifn. of, heat agglomeration in)
IT Cell
(nucleic acids and other heat-agglomeration-resistant water-sol.

miscellaneous 67-63-0, Isopropanol, uses and miscellaneous 108-95-2, Phenol, uses and miscellaneous 151-21-3, **Sodium dodecyl sulfate**, uses and miscellaneous 9001-63-2, Lysozyme 9002-93-1, Triton X-100
RL: ANST (Analytical study)
(in isolation and purifn. of nucleic acids and other heat-agglomeration-resistant water-sol. nitrogen-contg. org. compds.)

21 ANSWER 1 OF 5 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.DUPLICATE 1
AN 1995:29827 BIOSIS
DN PREV199598044127
TI Cholate and pH **reduce** interference by **sodium dodecyl sulfate** in the determination of DNA with Hoechst.
AU Bester, M. J.; Potgieter, H. C.; Vermaak, W. J. H.
CS Dep. Chem. Pathol., Univ. Pretoria, P.O. Box 2034, Pretoria 0001 South Africa
SO Analytical Biochemistry, (1994) Vol. 223, No. 2, pp. 299-305.
ISSN: 0003-2697.
DT Article
LA English
AB The use of the fluorescent dye 33258 Hoechst (Hoe) to quantitatively determine DNA in **cell** culture in the presence of **lysing** agents like **sodium dodecyl sulfate** (SDS) is limited by the masking effect of high levels of nonspecific fluorescence, caused by the binding of Hoe to micelles. The masking effect can be **reduced** substantially by increasing the concentration of the counterion, the addition of cholate, or the pH of the buffer. An optimized method was developed, combining the antimasking effects of sodium chloride, cholate, and pH to accurately determine DNA concentrations as low as 15 ng/ ml in the presence of up to 6.9 mm (0.2%) SDS. The effectiveness of SDS in **cell** dissolution can now be combined with the specificity and sensitivity of Hoe to determine **cellular** DNA.
CC Cytology and Cytochemistry - Animal *02506
Biochemical Methods - Nucleic Acids, Purines and Pyrimidines *10052
Biochemical Studies - General *10060
Biochemical Studies - Nucleic Acids, Purines and Pyrimidines *10062
Biochemical Studies - Minerals 10069
Metabolism - Minerals *13010
BC Animalia - Unspecified *33000
IT Major Concepts
Biochemistry and Molecular Biophysics; **Cell** Biology; Metabolism; Methods and Techniques
IT Chemicals & Biochemicals
CHOLATE; **SODIUM DODECYL SULFATE**; SODIUM CHLORIDE
IT Miscellaneous Descriptors
ANALYTICAL METHOD; **CELLULAR** CONCENTRATION; MASKING EFFECT; MICELLE; SODIUM CHLORIDE; 33258 HOECHST
ORGN Super Taxa
Animalia - Unspecified: Animalia
ORGN Organism Name
animal (Animalia - Unspecified); Animalia (Animalia - Unspecified)
ORGN Organism Superterms
animals
RN 81-25-4 (CHOLATE)
151-21-3 (**SODIUM DODECYL SULFATE**)
7647-14-5 (SODIUM CHLORIDE)

L6 ANSWER 5 OF 16 CAPLUS COPYRIGHT 2002 ACS
AN 1996:738071 CAPLUS
DN 126:4194
TI Method and reagent system for isolation, identification and/or analysis of leukocytes from **whole blood** samples
IN Ledis, Stephen L.; Crews, Harold R.; Fischer, Timothy J.; Sena, Ted
PA Coulter Electronics, Inc., USA
SO Can., 43 pp.
CODEN: CAXXA4
DT Patent
LA English
IC ICM G01N033-50
ICS G01N033-48; G01N015-12; G01N001-28
CC 9-2 (Biochemical Methods)
Section cross-reference(s): 13, 14
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	CA 1338603	A1	19960924	CA 1988-561207	19880311
	IL 85532	A1	19920329	IL 1988-85532	19880224
	WO 8807187	A1	19880922	WO 1988-US762	19880311
	W: AU, BR, DK, JP, KR, NO				
	RW: AT, BE, CH, DE, FR, GB, IT, LU, NL, SE				
	AU 8815417	A1	19881010	AU 1988-15417	19880311
	EP 305491	A1	19890308	EP 1988-903078	19880311
	EP 305491	B1	19960207		
	R: BE, CH, DE, FR, GB, IT, LI, NL, SE				
	JP 01502931	T2	19891005	JP 1988-502991	19880311
	JP 2796325	B2	19980910		
	ES 2010248	A6	19891101	ES 1988-749	19880311
	EP 627624	A1	19941207	EP 1994-113130	19880311
	EP 627624	B1	19970521		
	R: BE, CH, DE, FR, GB, IT, LI, NL, SE				
	US 5155044	A	19921013	US 1991-808041	19911212
	US 5731206	A	19980324	US 1996-630868	19960329
PRAI	US 1987-25303		19870313		
	US 1987-130911		19871210		
	EP 1988-903078		19880311		
	WO 1988-US762		19880311		
	US 1989-317147		19890228		
	US 1990-611378		19901113		
	US 1992-957543		19921006		
	US 1994-325531		19941018		

OS MARPAT 126:4194
AB A reagent system is disclosed for chem. treatment of a **whole-blood** sample, said reagent system comprising a 1st aq. soln. having a lytic reagent comprising a water-sol. compd. that at least partially dissocs. in aq. media to generate free protons and counterions, said 1st aq. soln. comprising a blood cell differentiation effective amt. of said lytic reagent. Said reagent system is characterized in that it effects the partitioning of said **whole blood** sample into 2 distinct fractions, an essentially intact leukocyte fraction and a **lysed-erythrocyte** fraction; said blood cell differentiation effective amt. of said lytic reagent, when added to the **whole blood** sample, effecting: (1) a decrease in the pH of the sample from its physiol. level to a pH in the range of from about 2.6 to about 4.0, while maintaining the osmolality of the sample at less than about 100 mOs; (2) rapid and essentially complete hemolysis of said **erythrocyte** fraction; and (3) subtle changes in said leukocyte fraction to enhance the ability of instrumentation to perform differential anal. and identification of at least 5 subpopulations of leukocytes, said subtle changes being effected while preserving said leukocyte fraction in its essentially native physiol. and/or immunochem. state.
ST blood leukocyte isolation identification lytic reagent
IT Cytometry
(flow; method and reagent for leukocyte isolation and identification and/or anal. in **whole blood**)
IT Antiserums
Blood analysis
Disease, animal
Erythrocyte
Hemolysis

biological studies 75-75-2, Methanesulfonic acid 77-92-9, Citric acid, biological studies 88-75-5, o-Nitrophenol 98-11-3, Benzenesulfonic acid, biological studies 98-47-5, m-Nitrobenzenesulfonic acid 100-02-7, p-Nitrophenol, biological studies 104-15-4, p-Toluenesulfonic acid, biological studies 106-48-9, p-Chlorophenol 110-15-6, Butanedioic acid, biological studies 554-84-7, m-Nitrophenol 594-45-6, Ethanesulfonic acid 767-00-0, p-Cyanophenol 7664-93-9, Sulfuric acid, biological studies
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BUU (Biological use, unclassified); BIOL (Biological study); USES (Uses)
(method and reagent for leukocyte isolation and identification and/or anal. in **whole blood**)

IT 144-55-8, Sodium bicarbonate, biological studies 497-19-8, Sodium carbonate, biological studies 7647-14-5, Sodium chloride (NaCl), biological studies 7757-82-6, Sodium sulfate, biological studies 26628-22-8, Sodium azide
RL: BUU (Biological use, unclassified); BIOL (Biological study); USES (Uses)
(method and reagent for leukocyte isolation and identification and/or anal. in **whole blood**)

L6 ANSWER 5 OF 16 CAPLUS COPYRIGHT 2002 ACS
AN 1996:738071 CAPLUS
DN 126:4194
TI Method and reagent system for isolation, identification and/or analysis of leukocytes from **whole blood** samples
IN Ledis, Stephen L.; Crews, Harold R.; Fischer, Timothy J.; Sena, Ted
PA Coulter Electronics, Inc., USA
SO Can., 43 pp.
CODEN: CAXXA4
DT Patent
LA English
IC ICM G01N033-50
ICS G01N033-48; G01N015-12; G01N001-28
CC 9-2 (Biochemical Methods)
Section cross-reference(s): 13, 14
FAN.CNT 1

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PI	CA 1338603	A1	19960924	CA 1988-561207	19880311
	IL 85532	A1	19920329	IL 1988-85532	19880224
	WO 8807187	A1	19880922	WO 1988-US762	19880311
	W: AU, BR, DK, JP, KR, NO				
	RW: AT, BE, CH, DE, FR, GB, IT, LU, NL, SE				
	AU 8815417	A1	19881010	AU 1988-15417	19880311
	EP 305491	A1	19890308	EP 1988-903078	19880311
	EP 305491	B1	19960207		
	R: BE, CH, DE, FR, GB, IT, LI, NL, SE				
	JP 01502931	T2	19891005	JP 1988-502991	19880311
	JP 2796325	B2	19980910		
	ES 2010248	A6	19891101	ES 1988-749	19880311
	EP 627624	A1	19941207	EP 1994-113130	19880311
	EP 627624	B1	19970521		
	R: BE, CH, DE, FR, GB, IT, LI, NL, SE				
	US 5155044	A	19921013	US 1991-808041	19911212
	US 5731206	A	19980324	US 1996-630868	19960329
PRAI	US 1987-25303		19870313		
	US 1987-130911		19871210		
	EP 1988-903078		19880311		
	WO 1988-US762		19880311		
	US 1989-317147		19890228		
	US 1990-611378		19901113		
	US 1992-957543		19921006		
	US 1994-325531		19941018		

OS MARPAT 126:4194
AB A reagent system is disclosed for chem. treatment of a **whole-blood** sample, said reagent system comprising a 1st aq. soln. having a lytic reagent comprising a water-sol. compd. that at least partially dissocs. in aq. media to generate free protons and counterions, said 1st aq. soln. comprising a blood cell differentiation effective amt. of said lytic reagent. Said reagent system is characterized in that it effects the partitioning of said **whole blood** sample into 2 distinct fractions, an essentially intact leukocyte fraction and a **lysed-erythrocyte** fraction; said blood cell differentiation effective amt. of said lytic reagent, when added to the **whole blood** sample, effecting: (1) a decrease in the pH of the sample from its physiol. level to a pH in the range of from about 2.6 to about 4.0, while maintaining the osmolality of the sample at less than about 100 mOs; (2) rapid and essentially complete hemolysis of said **erythrocyte** fraction; and (3) subtle changes in said leukocyte fraction to enhance the ability of instrumentation to perform differential anal. and identification of at least 5 subpopulations of leukocytes, said subtle changes being effected while preserving said leukocyte fraction in its essentially native physiol. and/or immunochem. state.
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IT Cytometry
(flow; method and reagent for leukocyte isolation and identification and/or anal. in **whole blood**)
IT Antiserums
Blood analysis
Disease, animal
Erythrocyte
Hemolysis

biological studies 75-75-2, Methanesulfonic acid 77-92-9, Citric acid, biological studies 88-75-5, o-Nitrophenol 98-11-3, Benzenesulfonic acid, biological studies 98-47-5, m-Nitrobenzenesulfonic acid 100-02-7, p-Nitrophenol, biological studies 104-15-4, p-Toluenesulfonic acid, biological studies 106-48-9, p-Chlorophenol 110-15-6, Butanedioic acid, biological studies 554-84-7, m-Nitrophenol 594-45-6, Ethanesulfonic acid 767-00-0, p-Cyanophenol 7664-93-9, Sulfuric acid, biological studies
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BUU (Biological use, unclassified); BIOL (Biological study); USES (Uses)
(method and reagent for leukocyte isolation and identification and/or anal. in **whole blood**)

IT 144-55-8, Sodium bicarbonate, biological studies 497-19-8, Sodium carbonate, biological studies 7647-14-5, Sodium chloride (NaCl), biological studies 7757-82-6, Sodium sulfate, biological studies 26628-22-8, Sodium azide
RL: BUU (Biological use, unclassified); BIOL (Biological study); USES (Uses)
(method and reagent for leukocyte isolation and identification and/or anal. in **whole blood**)

L6 ANSWER 8 OF 16 CAPLUS COPYRIGHT 2002 ACS
AN 1989:91671 CAPLUS
DN 110:91671
TI Method and reagents for the volumetric differentiation of blood cell types
IN Lapicola, James D.; Edmondson, Sherburne M., Jr.
PA Sequoia-Turner Corp., USA; Hematology Marketing Associates, Inc.
SO U.S., 8 pp. Division of U.S. Ser. No. 772,666, abandoned.
CODEN: USXXAM
DT Patent
LA English
IC ICM G01N033-50
NCL 436063000
CC 9-4 (Biochemical Methods)
Section cross-reference(s): 13, 14

FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI US 4745071	A	19880517	US 1986-914637	19861002
PRAI US 1985-772666		19850905		

OS MARPAT 110:91671

AB Improved reagents and methods for obtaining distinct differentiation of platelets, **erythrocytes**, and certain leukocyte subpopulations are disclosed. 1,3-Dimethylurea is a cell stabilizing agent for use in the blood diluent reagent. A diluent and a wetting agent (e.g. Diazopon) are combined to provide a hematol. analyzer detergent with the necessary attributes for an automatic analyzer. The **lysing** reagent is selective and intrinsically gentle and comprises an aq. soln. of a single quaternary ammonium salt. Leukocyte subpopulation volumetric differentiation in an automated system comprises mixing a **whole blood** sample with the diluent, adding the **lysing** agent extremely slowly, and analyzing the sample on an automatic **particle** analyzer to enumerate the lymphocyte and neutrophilic granulocyte subpopulations and to quant. evaluate the infrequent and rare leukocyte subpopulations. Automated hematol. anal. involved feeding a mixt. of **whole blood** and diluent, comprising Na2SO4 10.0, NaCl 4.2, 1,3-dimethylurea 1.0, 1-hydroxypyridine-2-thione 0.1, ADA buffer 1.4, NaOH 0.5 g, and water to 1 L, into a white cell counting bath; slowly adding **lysing** agent, comprising dodecyltrimethylammonium chloride (50% wt./vol.) 75, KCN 150 mg, and water to 1 L, to the counting bath; and analyzing the mixt. The normal leukocyte distribution had 76% neutrophils, 20% lymphocytes, and 4% infrequent leukocytes. A patient with a severe deficiency of lymphocytes had a distribution of 87%, 7%, and 6%, resp.

ST blood cell differentiation reagent; neutrophil lymphocyte infrequent leukocyte sepn; dimethylurea blood cell stabilization; dodecyltrimethylammonium chloride hematol

IT Bactericides, Disinfectants, and Antiseptics
Salts, biological studies
RL: BIOL (Biological study)
(blood diluent contg., in automated volumetric differentiation of blood leukocytes)

IT Quaternary ammonium compounds, biological studies
RL: ANST (Analytical study)
(**lysing** agent contg., in automated volumetric differentiation of blood leukocytes)

IT Leukocyte
Lymphocyte
Neutrophil
(no. of, automated volumetric detn. of, reagents for)

IT 96-31-1, 1,3-Dimethylurea
RL: ANST (Analytical study)
(as cell stabilizer in automated volumetric differentiation of blood leukocytes)

IT 1121-30-8, 1-Hydroxypyridine-2-thione 1132-61-2, MOPS 1310-73-2, Sodium hydroxide, biological studies 5625-37-6, PIPES 6976-37-0, BIS-TRIS 7365-44-8 7365-45-9, HEPES 7647-14-5, Sodium chloride, biological studies 7757-82-6, Sodium sulfate, biological studies 10191-18-1 26239-55-4
RL: ANST (Analytical study)
(blood diluent contg., in automated volumetric differentiation of blood leukocytes)

IT 151-50-8, Potassium cyanide

L6 ANSWER 8 OF 16 CAPLUS COPYRIGHT 2002 ACS
AN 1989:91671 CAPLUS
DN 110:91671
TI Method and reagents for the volumetric differentiation of blood cell types
IN Lapicola, James D.; Edmondson, Sherburne M., Jr.
PA Sequoia-Turner Corp., USA; Hematology Marketing Associates, Inc.
SO U.S., 8 pp. Division of U.S. Ser. No. 772,666, abandoned.
CODEN: USXXAM
DT Patent
LA English
IC ICM G01N033-50
NCL 436063000
CC 9-4 (Biochemical Methods)
Section cross-reference(s): 13, 14
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
US 4745071	A	19880517	US 1986-914637	19861002
PRAI US 1985-772666		19850905		

OS MARPAT 110:91671
AB Improved reagents and methods for obtaining distinct differentiation of platelets, **erythrocytes**, and certain leukocyte subpopulations are disclosed. 1,3-Dimethylurea is a cell stabilizing agent for use in the blood diluent reagent. A diluent and a wetting agent (e.g. Diazopon) are combined to provide a hematol. analyzer detergent with the necessary attributes for an automatic analyzer. The **lysing** reagent is selective and intrinsically gentle and comprises an aq. soln. of a single quaternary ammonium salt. Leukocyte subpopulation volumetric differentiation in an automated system comprises mixing a **whole blood** sample with the diluent, adding the **lysing** agent extremely slowly, and analyzing the sample on an automatic **particle** analyzer to enumerate the lymphocyte and neutrophilic granulocyte subpopulations and to quant. evaluate the infrequent and rare leukocyte subpopulations. Automated hematol. anal. involved feeding a mixt. of **whole blood** and diluent, comprising Na2SO4 10.0, NaCl 4.2, 1,3-dimethylurea 1.0, 1-hydroxypyridine-2-thione 0.1, ADA buffer 1.4, NaOH 0.5 g, and water to 1 L, into a white cell counting bath; slowly adding **lysing** agent, comprising dodecyltrimethylammonium chloride (50% wt./vol.) 75, KCN 150 mg, and water to 1 L, to the counting bath; and analyzing the mixt. The normal leukocyte distribution had 76% neutrophils, 20% lymphocytes, and 4% infrequent leukocytes. A patient with a severe deficiency of lymphocytes had a distribution of 87%, 7%, and 6%, resp.
ST blood cell differentiation reagent; neutrophil lymphocyte infrequent leukocyte sepn; dimethylurea blood cell stabilization; dodecyltrimethylammonium chloride hematol
IT Bactericides, Disinfectants, and Antiseptics
Salts, biological studies
RL: BIOL (Biological study)
(blood diluent contg., in automated volumetric differentiation of blood leukocytes)
IT Quaternary ammonium compounds, biological studies
RL: ANST (Analytical study)
(**lysing** agent contg., in automated volumetric differentiation of blood leukocytes)
IT Leukocyte
Lymphocyte
Neutrophil
(no. of, automated volumetric detn. of, reagents for)
IT 96-31-1, 1,3-Dimethylurea
RL: ANST (Analytical study)
(as cell stabilizer in automated volumetric differentiation of blood leukocytes)
IT 1121-30-8, 1-Hydroxypyridine-2-thione 1132-61-2, MOPS 1310-73-2, Sodium hydroxide, biological studies 5625-37-6, PIPES 6976-37-0, BIS-TRIS 7365-44-8 7365-45-9, HEPES 7647-14-5, Sodium chloride, biological studies 7757-82-6, Sodium sulfate, biological studies 10191-18-1 26239-55-4
RL: ANST (Analytical study)
(blood diluent contg., in automated volumetric differentiation of blood leukocytes)
IT 151-50-8, Potassium cyanide

L6 ANSWER 13 OF 16 CAPLUS COPYRIGHT 2002 ACS
AN 1979:164340 CAPLUS
DN 90:164340
TI Determination of a diagnostic indicator of a blood sugar condition, and a liquid chromatographic microcolumn
IN Acuff, Kenneth J.
PA Isolab, Inc., USA
SO U.S., 8 pp.
CODEN: USXXAM
DT Patent
LA English
IC G01N033-16
NCL 023230000B
CC 9-4 (Biochemical Methods)
Section cross-reference(s): 14

FAN.CNT 5

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	US 4142855	A	19790306	US 1977-856722	19771202
	DE 2851827	A1	19790613	DE 1978-2851827	19781130
	DE 2851827	C2	19830616		
	GB 2012068	A	19790718	GB 1978-46811	19781201
	GB 2012068	B2	19820217		
	GB 2011801	A	19790718	GB 1978-46813	19781201
	GB 2011801	B2	19820113		
	JP 54099496	A2	19790806	JP 1978-148000	19781201
	JP 62011308	B4	19870311		
	CH 648128	A	19850228	CH 1978-12325	19781201
PRAI	US 1977-856721		19771202		
	US 1977-856722		19771202		
	US 1977-856723		19771202		
	US 1977-856724		19771202		
	US 1977-856725		19771202		
	US 1978-932647		19780810		

AB A method for the detn. of the percent of Hb A1a-c relative to the total Hb content in blood samples as a diagnostic indicator of blood sugar levels is described, using a DEAE-cellulose anion-exchange microchromatog. system. The column bed of cellulose **particles** are equilibrated to pH 8.5 at 22.5.degree. using a tris-cyanide soln. consisting of 6.06 g tris (0.05 M), 0.10 g KCN (0.01%), and 0.10 g NaN3 (0.01%) as preservative. A **whole blood** sample is **lysed** and an **erythrocyte** hemolyzate prepd. The hemolyzate is introduced into the end of the column, eluted with the tris-cyanide soln., and the eluate measured spectrometrically. The remaining Hb fractions are successively eluted and these eluates measured spectrometrically. The percentage of the amt. of the 1st eluate relative to the amt. of the total hemolyzate is used as the diagnostic indicator. The method is useful in diabetes diagnosis.

ST Hb detn **erythrocyte** blood sugar; anion exchange chromatog Hb; diabetes diagnosis Hb **erythrocyte**

IT **Erythrocyte**
(Hb A1a-c detn. in, for diabetes diagnosis)

IT Diabetes mellitus
(Hb A2a-c detn. in **erythrocytes** in diagnosis of)

IT Hemoglobins
RL: ANT (Analyte); ANST (Analytical study)
(detn. of, in **erythrocytes**, for diabetes diagnosis)

IT Blood analysis
(glucose detn. in, Hb A1a-c detn. in **erythrocytes** in relation to)

IT 9013-34-7
RL: ANST (Analytical study)
(as anion-exchange chromatog. stationary phase, in Hb A1a-c detn. in **erythrocytes**)

IT 77-86-1 151-50-8
RL: ANST (Analytical study)
(buffer contg. tris and, in chromatog. of Hb A1a-c of **erythrocytes**)

IT 59979-42-9 59979-43-0 62572-11-6
RL: ANT (Analyte); ANST (Analytical study)
(detn. of, in **erythrocytes**, for diabetes diagnosis)

L6 ANSWER 13 OF 16 CAPLUS COPYRIGHT 2002 ACS
AN 1979:164340 CAPLUS
DN 90:164340
TI Determination of a diagnostic indicator of a blood sugar condition, and a liquid chromatographic microcolumn
IN Acuff, Kenneth J.
PA Isolab, Inc., USA
SO U.S., 8 pp.
CODEN: USXXAM
DT Patent
LA English
IC G01N033-16
NCL 023230000B
CC 9-4 (Biochemical Methods)
Section cross-reference(s): 14
FAN.CNT 5

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	US 4142855	A	19790306	US 1977-856722	19771202
	DE 2851827	A1	19790613	DE 1978-2851827	19781130
	DE 2851827	C2	19830616		
	GB 2012068	A	19790718	GB 1978-46811	19781201
	GB 2012068	B2	19820217		
	GB 2011801	A	19790718	GB 1978-46813	19781201
	GB 2011801	B2	19820113		
	JP 54099496	A2	19790806	JP 1978-148000	19781201
	JP 62011308	B4	19870311		
	CH 648128	A	19850228	CH 1978-12325	19781201
PRAI	US 1977-856721		19771202		
	US 1977-856722		19771202		
	US 1977-856723		19771202		
	US 1977-856724		19771202		
	US 1977-856725		19771202		
	US 1978-932647		19780810		

AB A method for the detn. of the percent of Hb A1a-c relative to the total Hb content in blood samples as a diagnostic indicator of blood sugar levels is described, using a DEAE-cellulose anion-exchange microchromatog. system. The column bed of cellulose **particles** are equilibrated to pH 8.5 at 22.5.degree. using a tris-cyanide soln. consisting of 6.06 g tris (0.05 M), 0.10 g KCN (0.01%), and 0.10 g NaN3 (0.01%) as preservative. A **whole blood** sample is **lysed** and an **erythrocyte** hemolyzate prepd. The hemolyzate is introduced into the end of the column, eluted with the tris-cyanide soln., and the eluate measured spectrometrically. The remaining Hb fractions are successively eluted and these eluates measured spectrometrically. The percentage of the amt. of the 1st eluate relative to the amt. of the total hemolyzate is used as the diagnostic indicator. The method is useful in diabetes diagnosis.

ST Hb detn **erythrocyte** blood sugar; anion exchange chromatog Hb; diabetes diagnosis Hb **erythrocyte**

IT **Erythrocyte**
(Hb A1a-c detn. in, for diabetes diagnosis)

IT Diabetes mellitus
(Hb A2a-c detn. in **erythrocytes** in diagnosis of)

IT Hemoglobins
RL: ANT (Analyte); ANST (Analytical study)
(detn. of, in **erythrocytes**, for diabetes diagnosis)

IT Blood analysis
(glucose detn. in, Hb A1a-c detn. in **erythrocytes** in relation to)

IT 9013-34-7
RL: ANST (Analytical study)
(as anion-exchange chromatog. stationary phase, in Hb A1a-c detn. in **erythrocytes**)

IT 77-86-1 151-50-8
RL: ANST (Analytical study)
(buffer contg. tris and, in chromatog. of Hb A1a-c of **erythrocytes**)

IT 59979-42-9 59979-43-0 62572-11-6
RL: ANT (Analyte); ANST (Analytical study)
(detn. of, in **erythrocytes**, for diabetes diagnosis)

L6 ANSWER 10 OF 16 CAPLUS COPYRIGHT 2002 ACS
AN 1981:546638 CAPLUS
DN 95:146638
TI Differential lymphoid-myeloid determination of leukocytes in **whole blood**
IN Ledis, Stephen L.; Chastain, David L., Jr.; Crews, Harold R.
PA Coulter Electronics, Inc., USA
SO U.S., 6 pp.
CODEN: USXXAM
DT Patent
LA English
IC G01N033-48; G01N033-72
NCL 023230000B
CC 9-13 (Biochemical Methods)
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
US 4286963	A	19810901	US 1979-96697	19791123

AB A lytic diluent and a method are described for rapidly **lysing** **erythrocytes** in **whole blood** for the differential detn. of lymphoid (lymphocytes) and myeloid (neutrophils, monocytes, eosinophils, and basophils) populations of leukocytes and for detn. of Hb, esp. in automatic **particle**-counting systems. The diluent contains .ltoreq.1 surface-active quaternary ammonium salt, substituted short-chain alkanols and polyhydroxy compds. as additives, and, when the reagent is used in lower concns., a reagent contg. a quaternary ammonium salt and an alkali metal cyanide, at alk. pH, may be added to complete chromogen formation. Thus, a stronger-concn., 1-reagent **lysing** reagent was prepd. by mixing, in 0.9% NaCl, 250 mg/L Cetrimide (hexadecyltrimethylammonium bromide) for efficient **erythrocyte lysis**, 200 mg/L Mytab (tetradecyltrimethylammonium bromide) for Hb conversion to chromogen, 0.6% 2-phenoxyethanol to improve leukocyte distribution and the Hb chromogen curve, 5% sorbitol to improve peak sepn., and 0.2% citric acid. The final soln. was at pH 4.0. Chromogen formation required 30-60 s for completion, depending on the additives used.

ST blood leukocyte Hb detn hemolysis reagent; **erythrocyte** hemolysis reagent; automated leukocyte differentiation reagent

IT Hemoglobins
RL: ANT (Analyte); ANST (Analytical study)
(detn. of, automated, hemolysis reagents for)

IT Basophil
Eosinophil
Leukocyte
Lymphocyte
Monocyte
Neutrophil
(differential detn. of, automated, hemolysis reagents for)

IT Hemolysis
(reagents for, for automated leukocyte differentiation and Hb detn.)

IT Quaternary ammonium compounds, compounds
RL: ANST (Analytical study)
(salts, hemolysis reagent contg., for automated leukocyte differential and Hb detn.)

IT 7558-79-4 7758-11-4
RL: ANST (Analytical study)
(buffer, hemolysis reagent contg., for automated leukocyte differentiation and Hb detn.)

IT 8044-71-1 50-70-4, uses and miscellaneous 122-99-6 151-50-8 1119-97-7
RL: ANST (Analytical study)
(hemolysis reagent contg., for automated leukocyte differentiation and Hb detn.)

L6 ANSWER 10 OF 16 CAPLUS COPYRIGHT 2002 ACS
AN 1981:546638 CAPLUS
DN 95:146638
TI Differential lymphoid-myeloid determination of leukocytes in **whole blood**
IN Ledis, Stephen L.; Chastain, David L., Jr.; Crews, Harold R.
PA Coulter Electronics, Inc., USA
SO U.S., 6 pp.
CODEN: USXXAM
DT Patent
LA English
IC G01N033-48; G01N033-72
NCL 023230000B
CC 9-13 (Biochemical Methods)
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
US 4286963	A	19810901	US 1979-96697	19791123

AB A lytic diluent and a method are described for rapidly **lysing** **erythrocytes** in **whole blood** for the differential detn. of lymphoid (lymphocytes) and myeloid (neutrophils, monocytes, eosinophils, and basophils) populations of leukocytes and for detn. of Hb, esp. in automatic **particle**-counting systems. The diluent contains .ltoreq.1 surface-active quaternary ammonium salt, substituted short-chain alkanols and polyhydroxy compds. as additives, and, when the reagent is used in lower concns., a reagent contg. a quaternary ammonium salt and an alkali metal cyanide, at alk. pH, may be added to complete chromogen formation. Thus, a stronger-concn., 1-reagent **lysing** reagent was prepd. by mixing, in 0.9% NaCl, 250 mg/L Cetrimide (hexadecyltrimethylammonium bromide) for efficient **erythrocyte lysis**, 200 mg/L Mytab (tetradecyltrimethylammonium bromide) for Hb conversion to chromogen, 0.6% 2-phenoxyethanol to improve leukocyte distribution and the Hb chromogen curve, 5% sorbitol to improve peak sepn., and 0.2% citric acid. The final soln. was at pH 4.0. Chromogen formation required 30-60 s for completion, depending on the additives used.

ST blood leukocyte Hb detn hemolysis reagent; **erythrocyte** hemolysis reagent; automated leukocyte differentiation reagent

IT Hemoglobins
RL: ANT (Analyte); ANST (Analytical study)
(detn. of, automated, hemolysis reagents for)

IT Basophil
Eosinophil
Leukocyte
Lymphocyte
Monocyte
Neutrophil
(differential detn. of, automated, hemolysis reagents for)

IT Hemolysis
(reagents for, for automated leukocyte differentiation and Hb detn.)

IT Quaternary ammonium compounds, compounds
RL: ANST (Analytical study)
(salts, hemolysis reagent contg., for automated leukocyte differential and Hb detn.)

IT 7558-79-4 7758-11-4
RL: ANST (Analytical study)
(buffer, hemolysis reagent contg., for automated leukocyte differentiation and Hb detn.)

IT 8044-71-1 50-70-4, uses and miscellaneous 122-99-6 151-50-8 1119-97-7
RL: ANST (Analytical study)
(hemolysis reagent contg., for automated leukocyte differentiation and Hb detn.)

L3 ANSWER 7 OF 13 CAPLUS COPYRIGHT 2003 ACS
AN 1985:200735 CAPLUS
DN 102:200735
TI Detection of antigens and antibodies in blood
PA Terumo Corp., Japan
SO Jpn. Kokai Tokkyo Koho, 3 pp.
CODEN: JKXXAF
DT Patent
LA Japanese
IC ICM G01N033-543
ICS A61K039-44
CC 9-2 (Biochemical Methods)
Section cross-reference(s): 15
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	JP 60047962	A2	19850315	JP 1983-154922	19830826
	JP 02051150	B4	19901106		
PRAI	JP 1983-154922		19830826		

AB A **whole blood** sample is treated with a reagent contg. hemolyzing agents (such as saponins) and then with antibody or antigen-sensitized carriers for the detection of blood antigens or antibodies by **agglutination** reaction. Hemolyzing agents cause **hemolysis** of erythrocytes which interfere with the **agglutination** reaction. Thus, polystyrene latex (0.117 .mu. diam.) was sensitized with human .gamma.-globulin and sensitized latex was mixed with 0.5% bovine serum albumin and 0.4% saponin to form a reagent for the detection of rheumatoid factor in **whole blood**.

ST blood rheumatoid factor detection; latex reagent rheumatoid factor detection; saponin latex reagent rheumatoid factor detection; antigen detn blood latex reagent; antibody detn blood latex reagent

IT Rheumatoid factors
RL: ANT (Analyte); ANST (Analytical study)
(detection of, in human **whole blood**, sensitized latex reagents contg. hemolyzing agents for)

IT Antibodies
Antigens
RL: ANT (Analyte); ANST (Analytical study)
(detn. of, in blood, latex reagent for)

IT Blood analysis
(rheumatoid factor detection in, of human, sensitized latex reagent contg. hemolyzing agent for)

IT Saponins
RL: ANST (Analytical study)
(sensitized latex reagents contg., for rheumatoid factor detection in **whole blood**)

IT Globulins, blood
RL: ANST (Analytical study)
(.gamma.-, polystyrene latex sensitized with, reagent contg. saponin and, for rheumatoid factor detection in **whole blood**)

IT 9003-53-6
RL: ANST (Analytical study)
(latex, globulin-sensitized, reagent contg. saponin and, for rheumatoid factor detection in **whole blood**)

⑲日本国特許庁(JP)　⑪特許出願公開

⑫公開特許公報(A)　昭60－47962

⑤Int.Cl.⁴	識別記号	庁内整理番号	㊸公開　昭和60年(1985)3月15日
G 01 N 33/543		7906－2G	
A 61 K 39/44		7043－4C	

審査請求　未請求　発明の数 2　(全3頁)

㊹発明の名称　血中の抗原または抗体量の測定方法およびそれに使用する試験液

㉑特　願　昭58－154922

㉒出　願　昭58(1983)8月26日

㉗発明者　伊藤　良孝　調布市小島町2丁目55番1号　調布南コーポラス805

㉑出願人　テルモ株式会社　東京都渋谷区幡ヶ谷2丁目44番1号

㉔代理人　弁理士　西村　公佑

明細書

1．発明の名称

血中の抗原または抗体量の測定方法およびそれに使用する試験液

2．特許請求の範囲

(1)全血検体に溶血剤と抗原または抗体感作担体浮遊液とを加え、その凝集反応を追跡することを特徴とする血中の抗原または抗体量の測定方法。

(2)溶血剤と抗原または抗体感作担体とを含有することを特徴とする血中の抗原または抗体量の測定方法に使用する試験液。

(3)溶血剤がサポニンである特許請求の範囲第2項記載の試験液。

3．発明の詳細な説明

Ⅰ．発明の背景

技術分野

本発明は、血中の抗原または抗体量の測定方法およびそれに使用する試験液に関するものである。

さらに詳しくは、本発明は、血中の抗原または抗体量を免疫反応に基づく凝集反応により測定する方法およびそれに使用する試験液に関するものである。

本発明は、慢性関節リウマチの診断等各種の免疫学的検査に利用される。

先行技術およびその問題点

従来、凝集反応により血液中の抗原または抗体量を測定する場合、検体中に赤血球が存在すると肉眼による凝集の判定が困難であることから検体として血清が用いられていた。しかし検体の個数が多い場合等は血清の調製に相当の手間と時間を要する。また血清の調製のため、本来検査に必要としない余分な量の血液を採取しなければならない。

Ⅱ．発明の目的

そこで本発明は、血清を調製する手間を省き、全血から直接抗原または抗体量を測定することができる方法を提供することを目的とする。

さらに本発明は、上記の測定方法に使用される

試験液を提供することを目的とする。

かかる目的を達成するため、本発明は、全血検体に溶血剤の抗原または抗体感作担体浮遊液とを加え、その凝集反応を追跡することを特徴とする血中の抗原または抗体量の測定方法からなる。

さらに本発明は、溶血剤と抗原または抗体感作担体とを含有する上記測定方法に使用される試験液からなる。

さらに本発明は、溶血剤がサポニンである上記試験液からなる。

Ⅲ．発明の具体的説明

本発明の方法は、採取した全血検体に溶血剤と抗原または抗体感作担体浮遊液とを加え、その凝集反応を追跡することによって実施される。

上記方法において溶血剤としてはサポニンや各種の界面活性剤が使用される。溶血剤は凝集反応に先立って予め全血に加え、赤血球を溶解してもよく、あるいは、抗原または抗体感作担体浮遊液に約0.2 ～ 2%の濃度で加えておき、凝集反応の際に赤血球を溶解させてもよい。抗原または抗体感作担体としては、ラテックス樹脂、無機吸着剤、変品処理した固定赤血球等従来公知のものが特に限定なく使用されうる。

凝集反応の追跡は常法に従って行なわれる。即ち、全血1滴をスライドグラス上に滴下し、これに溶血剤および抗原または抗体感作担体浮遊液の1滴を加え木の棒でよく混和し、およそ20×25㎜ぐらいにひろげる。スライドグラスを両手にもち、1分間ゆり動かした後凝集の有無を肉眼で判定する。その際赤血球は溶解しているので凝集判定の阻げにならない。

次に実施例を示して本発明をさらに具体的に説明する。

実施例

（1）リューマチ因子（RF）検出用ヒトガンマーグロブリン感作ラテックスの作成

グリシン－塩化ナトリウム緩衝液（pH8.2）（以下GNBと略称する）にポリスチレンラテックス（粒径0.117 μ）を固形分2.0 %となるように加えて懸濁させる。一方、GNBに対して透析したヒトガンマーグロブリンを10㎎/㎖となるようにGNBに溶かす。両液を体積比1：1で混合し、50℃で1時間加温した。得られた液をGNBで遠心洗浄（17,000rpm、10分間）し、これに牛血清アルブミン0.5 %、サポニン0.4 %を含むGNBを加えて0.4 %感作ラテックス浮遊液を作成した。以上の条件では感作蛋白濃度は10～100 μgN/㎖、ラテックス粒子密度は4.53×10^{8}個／㎖となり、ラテックス粒子1個当たり75,000個のガンマーグロブリン分子が結合すると概算された。

（2）スライド凝集反応

上記（1）で得られた感作ラテックス1滴（約0.02～0.03㎖）および血液または血清1滴を反応用スライドグラス上でよく混ぜ合わせ、直径約2㎝程度にひろげて凝集反応を行なった。スライドグラスを前後にゆり動かしながら1分後に凝集の有無、程度を次の判定基準に従い判定した。結果を表1に示す。

陽性（＋）

液全体に凝集塊が極めて多く、凝集していることが肉眼ではっきり認められる。

陰性（－）

肉眼では全く凝集が認められない。

判定不能（？）

ラテックスの凝集が判然としない。

陽性コントロール

慢性関節リウマチ（RA）コントロール血清

（凝集力価160）

陰性コントロール

健常人血清

上記コントロール血清と健常人濃厚赤血球とをそれぞれ再構成し（ヘマトクリット値40%）、被検体とした。

表1

	感作ラテックス 被検体	サポニン添加 感作ラテックス	サポニン無添加 感作ラテックス
陰性 コントロール	健常人血清	−	−
	全血（抗凝固剤無）	−	?
	全血（ヘパリン血）	−	?
	全血（ＡＣＤ血）	−	?
陽性 コントロール	ＲＡ血清	+	+
	全血（抗凝固剤無）	+	?
	全血（ヘパリン血）	+	?
	全血（ＡＣＤ血）	+	?

表1から、サポニン添加感作ラテックス試験液においては、検体として全血を使用しても判定が明瞭であり特異性に優れていることが明らかである。

Ⅳ．発明の具体的作用効果

本発明によれば、全血から直接抗原または抗体量を測定することができる血中の抗原または抗体量測定方法が提供される。

本発明の方法においては前述した如く溶血剤が使用され、赤血球が溶解するので検体として全血を用いても凝集の有無判定は容易である。従って従来の測定法におけるように、凝集試験のために血清を調製する必要がなく、測定操作が簡略化される。

さらに本発明によれば、上記の測定法に好適に使用される試験液が提供される。本発明の試験液は、溶血剤と抗原または抗体感作担体とを含有しているので、これを全血と混合するだけで赤血球が溶け、凝集の判定が容易に行なわれる。

⑲日本国特許庁(JP)　⑪特許出願公開

⑫公開特許公報(A)　平2-51150

⑤Int.Cl.⁵　識別記号　庁内整理番号　⑬公開　平成2年(1990)2月21日
G 03 C 7/38　7915-2H

審査請求　未請求　請求項の数 1　(全25頁)

⑭発明の名称　新規なシアンカプラーを含有するハロゲン化銀写真感光材料

⑳特　願　昭63-202270
㉒出　願　昭63(1988)8月12日

㉑発明者　増川　豊明　東京都日野市さくら町1番地　コニカ株式会社内
㉑発明者　内田　卓　東京都日野市さくら町1番地　コニカ株式会社内
㉑発明者　中山　憲卓　東京都日野市さくら町1番地　コニカ株式会社内
㉑出願人　コニカ株式会社　東京都新宿区西新宿1丁目26番2号

明　細　書

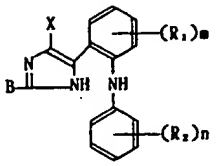
1．発明の名称

新規なシアンカプラーを含有するハロゲン化銀写真感光材料

2．特許請求の範囲

支持体上に少なくとも1層のハロゲン化銀乳剤層を有するハロゲン化銀写真感光材料において、前記ハロゲン化銀乳剤層の少なくとも1層が下記一般式〔I〕で表されるシアンカプラーの少なくとも1種を含有することを特徴とするハロゲン化銀写真感光材料。

一般式〔I〕



〔式中、Bは炭素原子、窒素原子、酸素原子又は硫黄原子を介してイミダゾール環に結合した有機基を表す。R_1及びR_2は各々1価の置換基を表し、mは0～4、nは0～5の整数を表す。m，nが2以上のときR_1，R_2は各々、異なった2つ以上の基を表してもよい。

Xは発色現像主薬の酸化体とカップリングする際に離脱しうる基を表す。〕

3．発明の詳細な説明

〔産業上の利用分野〕

本発明は新規なシアンカプラーを含有するハロゲン化銀写真感光材料に関する。

〔発明の背景〕

ハロゲン化銀カラー写真感光材料におけるシアンカプラーとしては、従来フェノール類又はナフトール類が多く用いられ、これらについては例えば米国特許2,369,929号、同2,474,293号等に記載されている。

しかし、フェノール類及びナフトール類から得られるシアン色素画像には、色再現上大きな問題があった。すなわち、これらシアン発色色素は吸収スペクトルの短波側のキレが悪く、グリーン部又、一部ブルー部にも不要な吸収を持っている。カラーペーパーやカラーリバーサル感光材料にお

いては、適切な補正手段がないため、色再現性をかなり悪化させているのが現状である。

そこで本発明者等はEP-249,453号、特願昭62-134144号等で新規なシアンカプラーを提案した。これらのカプラーは、形成されるシアン発色色素の分光吸収特性が従来のフェノール及びナフトール型シアンカプラーよりも優れており、色再現性が大きく改良された。又、発色色素のモル吸光係数が高く、従来のカプラーに較べ銀量の大幅低減が可能になった。しかしながら、得られた色素画像の堅牢性が充分でなく、更に堅牢性の高い色素画像を形成するシアンカプラーの開発が望まれていた。

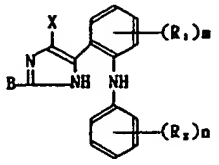
〔発明の目的〕

本発明の目的は、色再現性に優れ、かつ高い発色濃度を与えるシアンカプラーを含有するハロゲン化銀カラー写真感光材料を提供することにある。

〔発明の構成〕

本発明者等は鋭意研究の結果、支持体上に少なくとも1層のハロゲン化銀乳剤層を有するハロゲン化銀写真感光材料において、前記ハロゲン化銀乳剤層の少なくとも1層が下記一般式〔I〕で表されるシアンカプラーを含有するハロゲン化銀写真感光材料によって達成されることを見い出した。

一般式〔I〕



式中、Bは炭素原子、窒素原子、酸素原子又は硫黄原子を介してイミダゾール環に結合した有機基を表す。R_1及びR_2は各々1価の置換基を表し、mは0～4、nは0～5の整数を表す。m,nが2以上のときR_1,R_2は各々、異なった2つ以上の基を表してもよい。

Xは発色現像主薬の酸化体とカップリングする際に離脱しうる基を表す。

以下、本発明をより詳細に説明する。

上記一般式〔I〕において、Bで表される炭素原子を介した有機基としては、アルキル基（例えば、メチル、i-プロピル、t-ブチル、トリフルオロメチル、ベンジル、3-(4-アミノフェニル)プロピル、アリル、2-ドデシルオキシエチル、3-フェノキシプロピル、2-ヘキシルスルホニルエチル、3-〔4-(4-ドデシルオキシベンゼン)スルホンアミドフェニル〕プロピル、1-メチル-2-〔(2-オクチルオキシ-5-t-オクチルフェニル)スルホンアミドフェニル〕エチル、1-メチル-2-〔2-オクチルオキシ-5-(2-オクチルオキシ-5-t-オクチルフェニルスルホンアミド)フェニルスルホンアミド〕エチル、2-〔2-オクチルオキシ-5-(2-オクチルオキシ-5-t-オクチルフェニルスルホンアミド)フェニルスルホンアミド〕エチル等）、アリール基（例えば、フェニル、ナフチル、2,4-ジクロロフェニル、2-ヒドロキシ-5-メチルフェニル、2-アセトアミドフェニル、2-メタンスルホンアミドフェニル、2-ブタンアミドフェニル、2-(N,N-ジメチルスルファモイルアミノ)フェニル、2-(4-ドデシルオキシベンゼンスルホンアミド)フェニル、2-〔2-(2,4-ジ-t-アミルフェノキシ)ヘキサンアミド〕フェニル、2-(2-オクチルオキシ-5-t-オクチルフェニルスルホンアミド)フェニル、4-カルバモイルフェニル、4-シアノフェニル、4-カルボキシフェニル、4-エトキシカルボニルフェニル等）、複素環基（例えば、4-ピリジル、2-ベンゾイミダゾリル等）、シアノ基、カルボキシル基、アシル基、カルバモイル基、アルコキシカルボニル基、アリールオキシカルボニル基等が挙げられる。

窒素原子を介した有機基としては、アシルアミノ基（例えば、アセトアミド、ベンズアミド、2,4-ジ-t-アミルフェノキシアセトアミド、2,4-ジクロロベンズアミド等）、アルコキシカルボニルアミノ基（例えば、メトキシカルボニルアミノ、プロポキシカルボニルアミノ、t-ブトキシカルボニルアミノ等）、アリールオキシカルボニルアミノ基（例えば、フェノキシカルボニルアミノ）、スルホンアミド基（例えば、メタンスルホンアミド、オクタンスルホンアミド、ベンゼンスルホンアミド、4-ドデシルオキシベンゼンスルホンアミ

ド等）、アニリノ基（例えば、フェニルアミノ、2-クロロアニリノ、2-クロロ-4-テトラデカンアミドアニリノ等）、ウレイド基（例えば、N-メチルウレイド、N-ブチルウレイド、N-フェニルウレイド、N,N-ジブチルウレイド等）、スルファモイルアミノ基（例えば、N,N-ジエチルスルファモイルアミノ、N-フェニルスルファモイルアミノ等）、アミノ基（例えば、無置換アミノ、N-メチルアミノ、N,N-ジエチルアミノ等）、複素環基（例えば、3,5-ジメチル1-ピラゾリル、2,6-ジメチルモルホリノ等）等が挙げられる。

酸素原子を介した有機基としては、アルコキシ基（例えば、メトキシ、エトキシ、i-プロポキシ、ブトキシ、2,2,2-トリフルオロエトキシ、3,3,3-トリフルオロプロポキシ、2-クロロエトキシ、2-シアノエトキシ、2-ブタンスルホニルエトキシ等）、アリールオキシ基（例えば、フェノキシ、4-メトキシフェノキシ、2,4-ジクロロフェノキシ、4-(2-エチルヘキサンアミド)フェノキシ等）、シリルオキシ基（例えば、トリメチルシリルオキシ、ジメチルフェニルシリルオキシ、ジメチル-t-ブチルシリルオキシ等）、複素環オキシ基（例えば、テトラヒドロピラニルオキシ、3-ピリジルオキシ、2-(1,3-ベンゾイミダゾリル)オキシ等）等が挙げられる。

硫黄原子を介した有機基としては、アルキルチオ基（例えば、メチルチオ、エチルチオ、ブチルチオ、3-〔4-(4-ドデシルオキシベンゼン)スルホンアミドフェニル〕プロピルチオ、4-(2-ブトキシ-5-t-オクチルフェニルスルホンアミド)ベンジルチオ等）、アリールチオ基（例えば、フェニルチオ、2-ナフチルチオ、2,5-ジクロロフェニルチオ、4-ドデシルフェニルチオ、2-ブトキシ-5-t-オクチルフェニルチオ等）、複素環チオ基（例えば、2-ピリジルチオ、2-(1,3-ベンゾオキサゾリル)チオ基、1-ヘキサデシル1,2,3,4-テトラゾリル-5-チオ基、1-(3-N-オクタデシルカルバモイル)フェニル-1,2,3,4-テトラゾリル-5-チオ等）等が挙げられる。

R_1及びR_2で表される置換基は特に制限されないが、例えばハロゲン原子ならびにシアノ、ニトロ、カルボキシ、アルキル、アルコキシ、カルバモイル、スルファモイル、アシル、アシルオキシ、アルコキシカルボニル、$-NHCOR_3$、$-NHSO_2R_3$、

、$-NHCOOR_3$、

等の各基を挙げることができる。

R_1及びR_2で表されるアルキル基としては、炭素原子数1～22の直鎖または分岐のアルキル基が好ましく、例えばメチル、エチル、ブチル、ドデシル基等が挙げられる。これらのアルキル基はシクロヘキシル基等のシクロアルキル基も包含し、また置換されていてもよい。好ましい置換基としては、ハロゲン原子、ヒドロキシ基、カルボキシ基、シアノ基、スルホ基、炭素原子数1～22のアルコキシ基等が挙げられる。

アルコキシ基としては、炭素原子数1～22の直鎖又は分岐のアルコキシ基が好ましく、メトキシ、エトキシ、i-プロピルオキシ、オクチルオキシ、ドデシルオキシ基等が挙げられる。

カルバモイル基としては、エチルカルバモイル、ドデシルカルバモイル基のような非置換のアルキルカルバモイル基、ジエチルカルバモイル、ブチルオキシプロピルカルバモイル、ドデシルオキシプロピルカルバモイル基等の置換アルキルカルバモイル基が挙げられる。

又、スルファモイル基についても同様に、エチルスルファモイル、ジエチルスルファモイル、ドデシルスルファモイル基等の非置換アルキルスルファモイル基、ドデシルオキシプロピルスルファモイル基等の置換アルキルスルファモイル基が挙げられる。

アリールカルバモイル基としては、フェニルカルバモイル基や置換されたフェニルカルバモイル基が、アリールスルファモイル基としては、フェニルスルファモイル基や種々の置換されたフェニルスルファモイル基が挙げられる。

又、アセチル、ベンゾイル、ブタンスルホニル、ベンゼンスルホニル基等のアシル基、アセトキシ、ラウロイルオキシ、ブタンスルホニルオキシ基等

のアシルオキシ基、エトキシカルボニル、i-プロピルオキシカルボニル、2-エチルヘキシルオキシカルボニル基等のアルコキシカルボニル基が挙げられる。

$-NHCOR_3$基は炭素原子数1～22のアルキルアミド基を表し、非置換アルキルアミド基の代表例としては、アセトアミド、ブタンアミド、ラウリルアミド、ステアリルアミド基等が挙げられる。
又、シクロヘキサンカルボンアミド基の様な脂環式アミド基でもよく、又、2-エチルヘキサンアミド基の様な分岐構造でもよく、また不飽和結合を含んでいてもよい。

置換アルキルアミド基としては、モノクロルアセトアミド、トリクロロアセトアミド、パーフルオロブタンアミド基等のハロゲン置換アルキルアミド基やo-ペンタデシルフェノキシアセトアミド、α-(2,4-ジ-t-アミルフェノキシ)ペンタンアミド、α-(2,4-ジ-t-アシルフェノキシ)アセトアミド、o-クロルフェノキシミリスチン酸アミド基の如きフェノキシ置換アルキルアミド基等が挙げられる。

又、$-NHCOR_3$基はアリールアミド基を表し、代表的にはベンツアミド、ナフトアミド基等の非置換アリールアミド基が、また置換アリールアミド基としては、p-t-ブチルベンツアミド、p-メチルベンツアミド基等のアルキル置換ベンツアミド基、p-メトキシベンツアミド、o-ドデシルオキシベンツアミド基等のアルコキシ置換ベンツアミド基、p-アセトアミドベンツアミド、o-ラウロイルアミドベンツアミド、o-(2,4-ジ-t-アミルフェノキシアセトアミド)ベンツアミド基等のアミド置換ベンツアミド基、o-ヘキサデカンスルホンアミドベンツアミド、p-ブタンスルホンアミドベンツアミド基等のスルホンアミド置換ベンツアミド基等が代表的に挙げられる。

$-NHCOOR_3$基は炭素原子数1～22の置換又は非置換のアルコキシカルボニルアミノ基を表し、代表例としてはエトキシカルボニルアミノ、i-プロポキシカルボニルアミノ、オクチルオキシカルボニルアミノ、デシルオキシカルボニル、メトキシエトキシカルボニルアミノ基等が挙げられる。また、$-NHCOOR_3$基はアリールオキシカルボニル基も表し、この代表例としてはフェノキシカルボニル基が挙げられる。

$-NHCON\begin{matrix}R_3\\R_4\end{matrix}$基はジアルキルカルバモイルアミノ基を表し、代表的にはジメチルカルバモイルアミノ、ジエチルカルバモイルアミノ基等が挙げられる。

$-NHSO_2R_3$基はアルキルスルホンアミド基又はアリールスルホンアミド基を表す。

アルキルスルホンアミド基としては、メタンスルホンアミド、ブタンスルホンアミド、ドデカンスルホンアミド基等の炭素原子数1～22の非置換アルキルスルホンアミド基、ベンジルスルホンアミド基の様な置換アルキルスルホンアミド基等が挙げられる。

又、アリールスルホンアミド基としては、ベンゼンスルホンアミド、ナフタレンスルホンアミド基等の非置換アリールスルホンアミド基、又はp-トルエンスルホンアミド、2,4,6-トリメチルベンゼンスルホンアミド、p-ドデシルベンゼンスルホンアミド基等のアルキル置換ベンゼンスルホンアミド基、p-ドデシルオキシベンゼンスルホンアミド、ブチルオキシベンゼンスルホンアミド基等のアルコキシ置換ベンゼンスルホンアミド基などの置換アリールスルホンアミド基を挙げることができる。

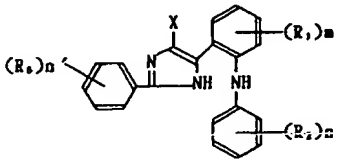
$-NHSO_2N\begin{matrix}R_3\\R_4\end{matrix}$基はスルファモイルアミノ基を表し、代表例としてはジメチルスルファモイルアミノ、ジブチルスルファモイルアミノ基等のジアルキルスルファモイルアミノ基が好ましい。

又、Xの表す発色現像主薬の酸化体との反応により脱離しうる基としては、例えばハロゲン原子（塩素、臭素、弗素等）およびヒドロキシル、アルコキシ、アリールオキシ、複素環オキシ、アシルオキシ、スルホニルオキシ、アルコキシカルボニルオキシ、アリールオキシカルボニル、アルキルオキザリルオキシ、アルコキシオキザリルオキシ、アルキルチオ、メルカプト、アリールチオ、

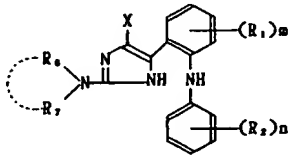
複素環チオ、アルコキシチオカルボニルチオ、アシルアミノ、置換アミノ、N原子で結合した含窒素複素環、スルホンアミド、アルキルオキシカルボニルアミノ、アリールオキシカルボニルアミノ、カルボキシル等の各基が挙げられるが、好ましくはハロゲン原子、特に塩素原子である。

一般式〔Ⅰ〕で表される化合物の中、代表的なものとして下記一般式〔Ⅱ〕、〔Ⅲ〕および〔Ⅳ〕が挙げられる。

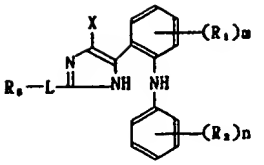
一般式〔Ⅱ〕



一般式〔Ⅲ〕



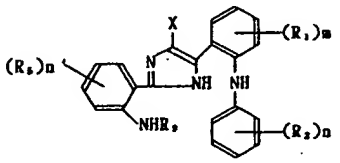
一般式〔Ⅳ〕



一般式〔Ⅱ〕～〔Ⅳ〕において、R_1, R_2, X, m及びnは前記一般式〔Ⅰ〕におけるR_1, R_2, m及びnと、それぞれ同義であり、R_3, R_4, R_5及びR_6は各々、置換基を表し、Lは酸素原子又は硫黄原子を表し、n′は0～5の整数を表す。n′が2以上のときR_6は異なった2つ以上の基を表してもよい。

又、一般式〔Ⅱ〕で表される化合物の中、更に好ましい化合物として一般式〔Ⅴ〕が挙げられる。

一般式〔Ⅴ〕



一般式〔Ⅴ〕において、R_1, R_2, R_3, X, m, n及びn′は一般式〔Ⅱ〕におけるR_1, R_2, R_3, X, m, n及びn′と、それぞれ同義である。R_3は水素原子、アルキル基、アリール基、

$-COR_3$、$-SO_2R_3$、$-CON\begin{matrix}R_3\\R_4\end{matrix}$、$-COOR_3$又は

$-SO_2N\begin{matrix}R_3\\R_4\end{matrix}$ を表す。

R_3で表されるアルキル基としては、炭素原子数1～32の直鎖または分岐のアルキル基が好ましく、シクロヘキシル基等のシクロアルキル基も包含する。また、これらのアルキル基は置換されていてもよく、好ましい置換基としてはハロゲン原子、ヒドロキシル基、カルボキシル基、シアノ基、スルホ基、炭素原子数1～22のアルコキシ基等を代表的に挙げることができる。

R_3で表されるアリール基としてはフェニル基が好ましく、フェニル基はニトロ基、アミド基、スルホンアミド基等で置換されてもよい。

スルホンアミド基等で置換されてもよい。

又、$-NHR_3$が$-NHCOR_3$基、$-NHCOOR_3$基、

$-NHCON\begin{matrix}R_3\\R_4\end{matrix}$ 基、$-NHSO_2R_3$基又は、

$-NHSO_2N\begin{matrix}R_3\\R_4\end{matrix}$ 基で表される場合、R_3及びR_4は一般式〔Ⅱ〕におけるR_3及びR_4と、それぞれ同義である。

又、一般式〔Ⅲ〕において、R_4又はR_7で表されるアルキル基、アリール基としては、前記一般式〔Ⅴ〕のR_3において説明したアルキル基、アリール基を具体的に挙げることができる。

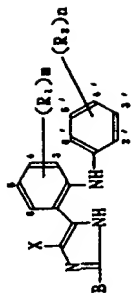
R_4とR_7が結合して形成される複素環は5員または6員のものが好ましく、これら複素環は置換基を有してもよく、更に炭素環と縮合していてもよい。

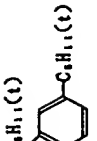
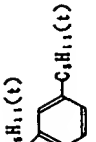
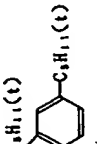
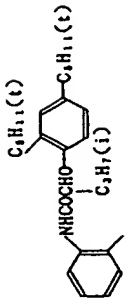
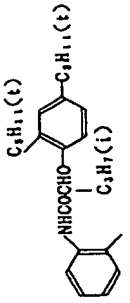
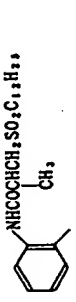
次に一般式〔Ⅳ〕において、R_5は水素原子、アルキル基、アリール基又は複素環基を表し、R_5で表されるアルキル基、アリール基としては、前

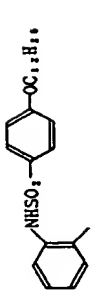
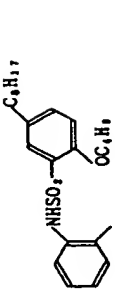
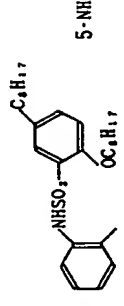
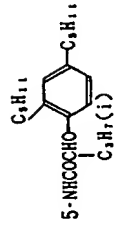
記一般式〔V〕において説明したアルキル基、アリール基を具体的に挙げることができる。

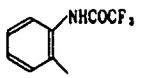
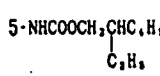
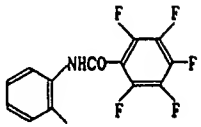
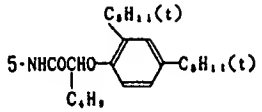
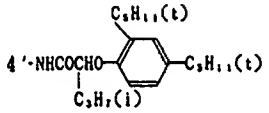
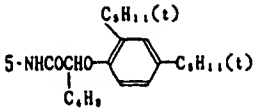
R_3で表される複素環基としては5員または6員のものが好ましく、具体的には2-ピリジル、4-ピリジル、2-ベンゾイミダゾリル、3,5-ジメチル-1-ピラゾリル、4-モルホリノ、3,5-ジメチル-2-フリル、2,4-ジメチル-5-チアゾリル、2-アセトアミド-4-メチル-5-ピリミジニル等の基が挙げられる。

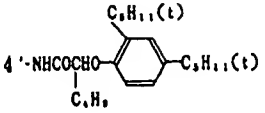
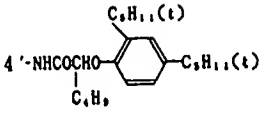
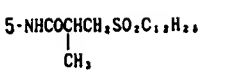
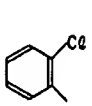
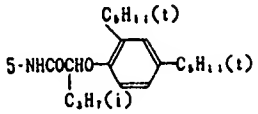
以下に本発明に用いられるシアンカプラーの代表的具体例を示すが、本発明はこれによって限定されない。

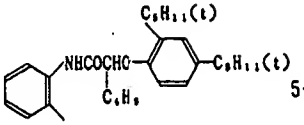
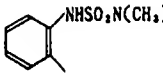
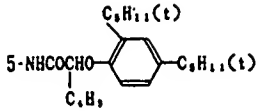
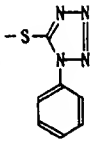
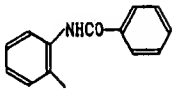
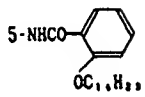
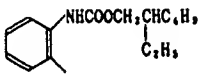
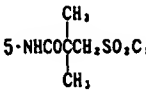


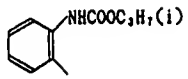
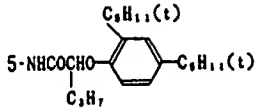
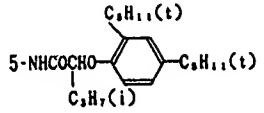
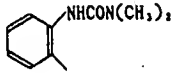
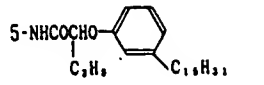
No.	X	B	R_1	R_3	m	n
1	H			—	1	0
2	Cℓ			—	1	0
3	Cℓ			2'—Br 4'—Br	1	2
4	H		—	—	0	0
5	Cℓ		—	—	0	0
6	H		—	—	0	0
7	Cℓ		—	—	0	0

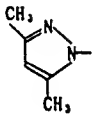
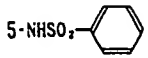
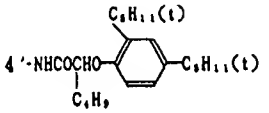
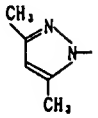
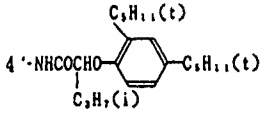
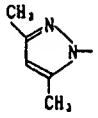
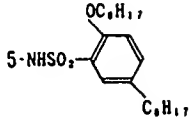
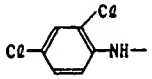
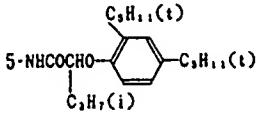
No.	X	B	R_1	R_3	m	n
8	H		5-NHCOCF$_3$	—	1	0
9	H		5-NHCOCH$_3$	—	1	0
10	Cℓ			—	1	0
11	Cℓ		5-NHCOC$_3$H$_7$(i)	—	1	0

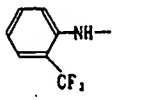
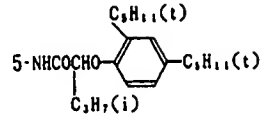
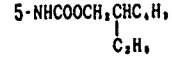
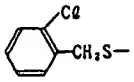
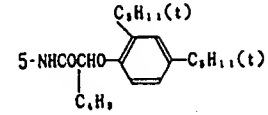
No.	X	B	R_1	R_2	m	n
20	Cℓ			—	1	0
21	Cℓ			4'-NHCOCH₃	1	1
22	H	CH_3O—	—		0	1
23	Cℓ	CH_3O—		2'-Cℓ 4'-Cℓ	1	2

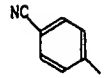
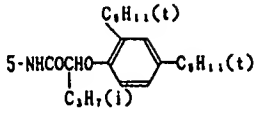
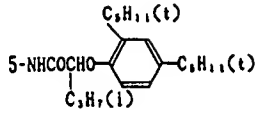
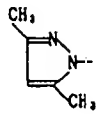
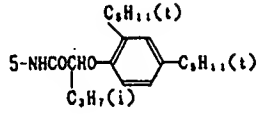
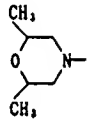
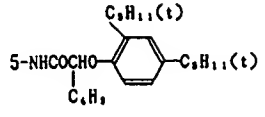
No.	X	B	R_1	R_2	m	n
24	H		—		0	1
25	H		—		0	1
26	Cℓ			—	1	0
27	H			2'-Br 4'-Br	1	2

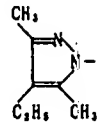
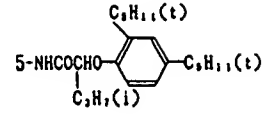
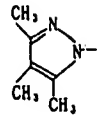
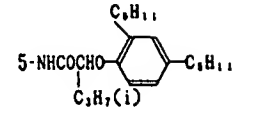
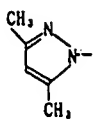
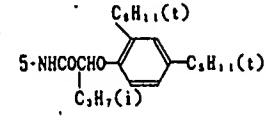
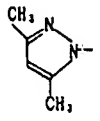
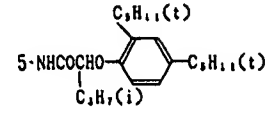
No.	X	B	R_1	R_2	m	n
12	Cℓ		5-NHCOC₃H₇(i)	4'-OCH₃	1	1
13	Cℓ			4'-CH₃	1	1
14				—	1	0
15	H			—	1	0

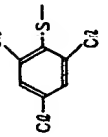
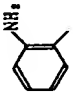
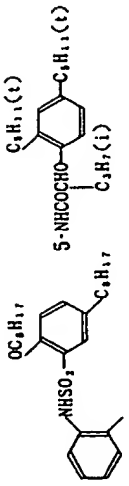
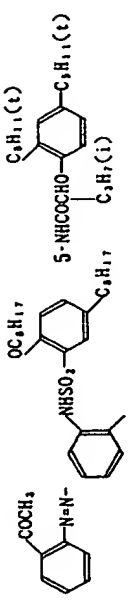
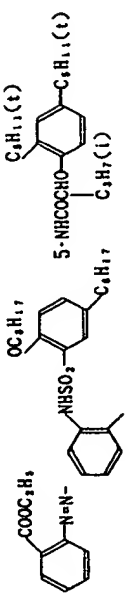
No.	X	B	R_1	R_2	m	n
16	H		5-NHSO₂C₁₆H₃₃	—	1	0
17	H			—	1	0
18	Cℓ			—	1	0
19	Cℓ			3'-CH₃	1	1

No.	X	B	R_1	R_2	m	n
36	Cℓ				1	1
37	Cℓ		5-NHSO$_2$C$_4$H$_9$		1	1
38	Cℓ			4'-CH$_3$	1	1
39	H			—	1	0

No.	X	B	R_1	R_2	m	n
40	H			—	1	0
41	H	(t)C$_8$H$_{17}$NH—		—	1	0
42	Cℓ	C$_{18}$H$_{33}$S—	5-NHCOOC$_3$H$_7$(i)	—	1	0
43	Cℓ			—	1	0

No.	X	B	R_1	R_2	m	n
28	H			2'-Br 4'-Br	1	2
29	H			—	1	0
30	H			2'-Br 4'-Br	1	2
31	H			—	1	0

No.	X	B	R_1	R_2	m	n
32	H			—	1	0
33	Cℓ			—	1	0
34	H			—	1	0
35	Cℓ			—	1	0

No.	X	B	R_1	R_2	m	n
44	Cℓ	$CH_3SO_2NHCH_2CH_2S-$	5-NHCOC$_2$H$_5$	4'-NHCOC$_{11}$H$_{23}$	1	1
45	Cℓ	(i)C$_3$H$_7$O-	5-NHCOCHO(C$_5$H$_{11}$(t)-C$_6$H$_4$-)(C$_2$H$_7$(i))	4'-Cℓ	1	1
46	Cℓ	$CH_3OCH_2CH(Cℓ)-$	5-NHCOCHO(C$_5$H$_{11}$(t)-C$_6$H$_4$-)(C$_3$H$_7$)	4'-Cℓ	1	1
47	Cℓ	$CℓCH_2-$	5-NHCOCHO(C$_5$H$_{11}$(t)-C$_6$H$_4$-)(C$_4$H$_9$)	2'-Cℓ 4'-Cℓ	1	2
48	Cℓ	C$_{12}$H$_{25}$S-	5-NHCOCHO(C$_5$H$_{11}$(t)-C$_6$H$_4$-)(C$_4$H$_9$)	4'-NHCOC$_3$H$_6$	1	1
49	H		5-NHCOCHO(C$_5$H$_{11}$(t)-C$_6$H$_4$-)(C$_4$H$_9$)	4'-NHCOCH$_3$	1	1
50	H		5-NHCOCHO(C$_5$H$_{11}$(t)-C$_6$H$_4$-)(C$_3$H$_7$(i))	2'-Br 4'-Br	1	2
51	H		5-NHCOCHO(C$_5$H$_{11}$(t)-C$_6$H$_4$-)(C$_3$H$_7$(i))	—	1	0
52	H		5-NHCOCHO(C$_5$H$_{11}$(t)-C$_6$H$_4$-)(C$_3$H$_7$(i))	—	1	0
53	H		5-NHCOCHO(C$_5$H$_{11}$(t)-C$_6$H$_4$-)(C$_3$H$_7$(i))	—	1	0
54	H		5-NHCOCHO(C$_5$H$_{11}$(t)-C$_6$H$_4$-)(C$_3$H$_7$(i))	—	1	0

本発明のカプラーの代表的合成例を示す。

合成例1（例示化合物30の合成）

2-クロル-5-ニトロアセトフェノンの合成

発煙硝酸(d＝1.52)80mℓを氷浴上で4℃に冷却し、ここにo-クロルアセトフェノン24.0gを4～7℃で約3時間かけて滴下した。4℃で1時間そのまま撹拌を続けた。

次いで反応液を氷にあけ析出した淡黄色の結晶を濾取した。この結晶をアルコールから再結晶し、22gの2-クロル-5-ニトロアセトフェノンを得た。融点59～61℃。

2-アセチル-4-ニトロジフェニルアミンの合成

2-クロル-5-ニトロアセトフェノン20g、アニリン20g及び無水炭酸カリウム20gを125℃～135℃で5時間加温撹拌する。

80℃位に冷えた所でアルコール80mℓを加え撹拌しながら放冷した。一晩このまま放置すると黄褐色の結晶が析出した。これを濾取、アルコールで洗い次いで水洗した。得られた粗結晶をアルコールから再結晶して11.0gの2-アセチル-4-ニトロジ

フェニルアミンを得た。

融点130～132℃。

2-アセチル-4-{2-(2',4'-ジ-t-アミルフェノキシ-i-ペンタンアミド)}ジフェニルアミンの合成

2-アセチル-4-ニトロジフェニルアミン10.2gとアルコール200mlに、Pd－C触媒0.5gを加え常圧で接触還元を行った。約3時間で2.8lの水素を吸収し反応が停止した(理論量1.35l)。反応液は濃い黄色を呈している。

触媒を濾過して除き、濾液を濃縮し、残留する黄褐色のオイルを真空ポンプでよく吸引して残留アルコールを除き、そのまま次の反応に用いた。

前記アミン化合物をアセトニトリル200mlに溶解し、ピリジン4.0gを加えた。ここに2,4-ジ-t-アミルフェノキシ-i-ペンタノイルクロライド14.1gをアセトニトリル50mlに溶解したものを流入する。発熱して結晶が出るが撹拌していると暫くして溶解する。これを2時間煮沸還流した後、濃縮した。

残留飴状物質を酢酸エチルで抽出し、酢酸エチル層を希塩酸で洗滌し、水洗した後、硫酸マグネシウムで脱水した。

酢酸エチル層で濃縮し、残留オイルをカラムクロマトにより精製した。黄色のアモルファス固体22.0gを得た。

例示化合物30の合成

2-アセチル-4-{2-(2',4'-ジ-t-アミルフェノキシ-i-ペンタンアミド)}ジフェニルアミン11.1gをクロロホルム150mlに溶解した。ここに室温で臭素9.85gを約3時間かけて滴下した。更に室温で30分、その後、温度を40～50℃に上げて30分保ち反応を完結させた後、濃縮した。残った黄褐色オイルをクロロホルム200mlに再溶解し、ここに撹拌しながら3,5-ジメチルピラゾールカルボアミジン＊(16.5gの硝酸塩から製した)のクロロホルム溶液を加え10時間煮沸還流した。結晶が析出してくるが、これは3,5-ジメチルピラゾールカルボアミジン硝酸塩なので反応後、濾過で除き、濾液を濃縮した。残ったオイル分をカラムクロマトで分離精製して13.4gの例示化合物30をアモルファス固体として得た。

このものはマススペクトルによりM⁺＝818を示し、前記構造であることが確認された。

＊ 3,5-ジメチルピラゾールカルボアミジン硝酸塩16.5gに50mlの水に加えて殆ど溶解させ後、撹拌しながら33gの水酸化カリウムを50mlの水に溶解した溶液を加えた。フリーのカルボアミジンがオイルとして遊離するので、これをクロロホルムで抽出する。クロロホルム抽出液を無水硫酸マグネシウムで脱水し、溶液をそのまま反応に用いた。

合成例2（例示化合物34の合成）

化合物例30 5.92gにアルコール160ml、トリエチルアミン1.82g、ラネーNi触媒を加えて常圧で32時間接触還元を行った。反応が進むにつれて白い沈殿が生成してくる。反応後、この白い結晶を濾取しアルコールで洗滌した。ラネーNi触媒と分けるために、得られた結晶を希アンモニア水溶液で処理し酢酸エチルで抽出した。酢酸エチル層を無水硫酸マグネシウムで脱水した後、濃縮した。残った灰白色の固体をアセトニトリルから再結晶して2.4gの例示化合物34を得た。

融点184.5～186.5℃。

合成例3（例示化合物35の合成）

前記例示化合物34 2.0gを、酢酸エチル30mlとジメチルホルムアミド50mlに溶解し、N-クロルコハク酸イミド0.44gを加え室温で3日間反応させた。水にあけ、酢酸エチルで抽出し、酢酸エチル層を水洗した後、無水硫酸マグネシウムで脱水後、濃縮した。残留淡褐色オイルをカラムクロマトにより精製した所、淡褐色のアモルファス固体1.1gを得た。

このものはマススペクトルによりM⁺＝694を示し、目的物であることが確認された。

合成例4（例示化合物50の合成）

例示化合物30の合成における3,5-ジメチルピラゾールカルボアミジンに代えて6.76gのo-ニトロベンツアミジンを用い、同様に反応させカラムクロマトで精製することにより2-(o-ニトロフェニル)-4-{2-(2',4'-ジブロムアニリノ)-5-{2'-(2",

4″-ジ-t-アミルフェノキシ-i-ペンタンアミド)]フェニル)イミダゾールを赤褐色アモルファス固体として得た。収量8.7g。

合成例5（例示化合物51の合成）

前記2-(o-ニトロフェニル)-4-[2-(2′,4′-ジブロムアニリノ)-5-[2′-(2″,4″-ジ-t-アミルフェノキシ-i-ペンタンアミド)]フェニル)イミダゾール7.0gにテトラヒドロフラン60mℓ、エタノール60mℓ、Pd-C触媒0.6gを加え常圧で接触還元を行った。約5日を要して反応が終了した。触媒を濾過で除き、濾液を濃縮して灰白色の固体5.46gを得た。

このものはマススペクトルによりM⁺=657を示し、2-(o-アミノフェニル)-4-[2-アニリノ-5-[2′-(2″,4″-ジ-t-アミルフェノキシ-i-ペンタンアミド)]フェニル]イミダゾールの構造を支持した。

合成例6（例示化合物52の合成）

上記例示化合物51のアミン4.2gにアセトニトリル40mℓを加え、更に2-オクチルオキシ-5-オクチルベンゼンスルホニルクロライド2.52gを加え、撹拌した。これにピリジン0.6gを滴下した後、室温で約3時間反応させた。

水を加えて酢酸エチルで抽出した。希塩酸、次いで水、更に希アンモニア水で酢酸エチル層を洗浄した後、再び水洗し、硫酸マグネシウムで脱水した。酢酸エチル層を濃縮し、残留油状物をアセトニトリルから再結晶して白色結晶3.78gを得た。融点184～186℃。

合成例7（例示化合物10の合成）

合成例6で得られた例示化合物52 3.28gを酢酸エチル100mℓに溶解し、ここにN-クロルコハク酸イミド0.52gを撹拌しながら加え溶解させた。3週間そのまま室温で放置した後、水を加え酢酸エチル層を分離した。酢酸エチル層を脱水後、濃縮し、3.5gの油状物を得た。カラムクロマトにより精製し、2.1gとなった油状物を真空ポンプで吸収した所、アモルファス固体を得た。

このものはマススペクトルによりM⁺=1071を示し化合物10であることが確認された。

合成例8（例示化合物17の合成）

合成例6において2-オクチルオキシ-5-オクチルベンゼンスルホニルクロライドの代りに0.82gのクロル蟻酸エチルを用いた他は同様に反応させた。カラムクロマトにより精製した所3.6gの油状物を得た。このものはマススペクトルによりM⁺=729を示し、例示化合物17の構造が支持された。

本発明のハロゲン化銀写真感光材料においては、本発明のシアンカプラーをハロゲン化銀1モル当り10～300g添加することが好ましいが、必要に応じて適宜変更することができる。

本発明のカプラーは2種以上を組み合わせて用いることができ、また他の種類のシアンカプラーと併用することもできる。

本発明のカプラーは、固体分散法、ラテックス分散法、水中油滴乳化分散法等、種々の方法を用いてハロゲン化銀写真感光材料へ添加することができる。例えば水中油滴乳化分散法は、カプラー等の疎水性添加物を通常、トリクレジルホスフェート、ジブチルフタレート等の沸点約150℃以上の高沸点有機溶媒に、必要に応じ酢酸エチル、ブロピオン酸ブチル等の低沸点および／または水溶性有機溶媒を併用して溶解し、ゼラチン水溶液などの親水性バインダー中に界面活性剤を用いて乳化分散した後、目的とする親水性コロイド層中に添加すればよい。

本発明のハロゲン化銀写真感光材料は、例えばカラーのネガ、ポジおよびリバーサルフィルムならびにカラー印画紙などに適用することができる。

このカラーフィルムを初めとする本発明のハロゲン化銀写真感光材料は、減色法色再現を行うために写真用カラーとして、本発明のマゼンタ、シアンカプラーおよびイエローカプラーを、それぞれ含有する緑感性、赤感性および青感性の乳剤層ならびに非感光性層が支持体上に適宜の層数および層順で積層した構造を有しているが、該層数および層順は重点性能、使用目的によって適宜変更してもよい。

本発明のハロゲン化銀写真感光材料に用いられるハロゲン化銀乳剤には、ハロゲン化銀として臭化銀、沃臭化銀、沃塩化銀、塩臭化銀および塩化銀等の通常のハロゲン化銀乳剤に使用される任意

のものを用いることができる。

ハロゲン化銀乳剤は、硫黄増感法、セレン増感法、還元増感法、貴金属増感法などにより化学増感される。また、写真業界において増感色素として知られている色素を用いて所望の波長域に光学増感できる。

本発明のハロゲン化銀写真感光材料には、色カブリ防止剤、硬膜剤、可塑剤、ポリマーラテックス、紫外線吸収剤、ホルマリンスカベンジャー、媒染剤、現像促進剤、現像遅延剤、蛍光増白剤、マット剤、滑剤、帯電防止剤、界面活性剤等を任意に用いることができる。

本発明においては、当業界で用いられる任意の処理を行うことができ、例えば発色現像処理、漂白、定着あるいは漂白定着、安定、水洗、停止等の処理を行うことができる。

〔発明の具体的効果〕

本発明のハロゲン化銀写真感光材料は、新規なシアンカプラーを含有するので形成されるシアン色素の分光吸収特性が良好であり、すなわち短波長側の切れが良く、グリーン部およびブルー部に不整吸収が少なく、色再現性に優れている。また、シアン色素の発色濃度が高い、つまり本発明のシアンカプラーが高発色性であるので、薄膜化等による鮮鋭性の向上が可能となる。更に形成色素の耐熱、耐光性が著しく改善される。

〔実施例〕

以下に本発明の具体的実施例を述べるが、本発明の実施の態様はこれらに限定されない。

実施例－1

表－1に示したシアンカプラー0.0126モルに対し、ジブチルフタレート3gを加え、更に酢酸エチル18gを加えた混合溶液を60℃に加熱して溶解した後、これをアルカノールB（アルキルナフタレンスルホネート、デュポン社製）の5%水溶液10mlを含む5%ゼラチン水溶液100mlに混合し、超音波分散機で乳化分散して分散液を調製した。

次にシアンカプラーが銀に対して10モル%になる様に前記分散液を沃臭化銀乳剤（沃化銀6モル%含有）に添加し、更に硬膜剤として、1,2-ビス(ビニルスルホニル)エタンをゼラチン1g当り12mgの割合で加えたのち、下引された透明な三酢酸セルロースフィルム支持体上に塗布銀量が18mg/100cm²になるように塗布した。かくして得られた各ハロゲン化銀写真感光材料を常法によりウェッジ露光した後、下記の現像処理を施した。

〔処理工程〕

処理工程(38℃)	処理時間
発色現像	3分15秒
漂　白	6分30秒
水　洗	3分15秒
定　着	6分30秒
水　洗	3分15秒
安定化	1分30秒

処理工程において使用される処理液組成は下記の通りである。

〔発色現像液組成〕

4-アミノ-3-メチル-N-エチル-N-(β-ヒドロキシエチル)アニリン硫酸塩	4.75g
無水亜硫酸ナトリウム	4.25g
ヒドロキシルアミン・1/2硫酸塩	2.0g
無水炭酸カリウム	37.5g
臭化ナトリウム	1.3g
ニトリロ三酢酸3ナトリウム塩(1水塩)	2.5g
水酸化カリウム	1.0g

水を加えて1lとし、水酸化カリウムを用いてpH 10.0に調整する。

〔漂白液組成〕

エチレンジアミン四酢酸鉄アンモニウム塩	100g
エチレンジアミン四酢酸2アンモニウム塩	10g
臭化アンモニウム	150g
氷酢酸	10ml

水を加えて1lとし、アンモニア水を用いてpH 6.0に調整する。

〔定着液組成〕

チオ硫酸アンモニウム

（50％水溶液）　　162mℓ

無水亜硫酸ナトリウム　　12.4g

水を加えて1ℓとし、酢酸を用いてpH6.5に調整する。

〔安定化液組成〕

ホルマリン（37％水溶液）　　5.0mℓ

コニダックス

（コニカ株式会社製）　　7.5mℓ

水を加えて1ℓとする。

かくして得られたハロゲン化銀写真感光材料のカブリ、感度S，およびシアン色素画像の最大濃度Dmaxを測定した。

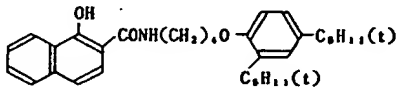
結果を表－1に示す。

表－1

試料 No.	カプラー	カブリ	S，	Dmax
1（比　較）	C－1*	0.14	100	1.45
2（本発明）	例示1	0.12	108	1.63
3（　〃　）	〃 4	0.13	107	1.65
4（　〃　）	〃 9	0.14	110	1.70
5（　〃　）	〃 29	0.14	108	1.68
6（　〃　）	〃 30	0.13	111	1.75
7（　〃　）	〃 34	0.11	113	1.75
8（比　較）	C－2*	0.25	130	2.10
9（本発明）	例示2	0.20	150	2.15
10（　〃　）	〃 5	0.23	163	2.20
11（　〃　）	〃 10	0.22	170	2.28
12（　〃　）	〃 18	0.24	165	2.25
13（　〃　）	〃 23	0.24	185	2.30
14（　〃　）	〃 35	0.23	160	2.20
15（　〃　）	〃 42	0.21	162	2.25

＊比較カプラー

C－1



C－2

表－1から本発明のカプラーは従来のカプラーより、いずれも高発色性であることがわかる。

実施例－2

（ハロゲン化銀乳剤の調製）

中性法、同時混合法により、表－2に示す6種類のハロゲン化銀乳剤を調製した。

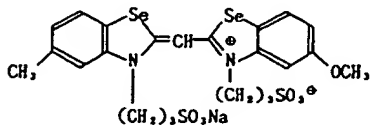
表－2

乳剤No.	AgCℓ モル%	AgBr モル%	平均粒径μm	化学増感剤	分光増感色素
Em－1	99.5	0.5	0.67	チオ硫酸	SD－1*3
Em－2	99.5	0.5	0.46	ナトリウム*1	SD－2*4
Em－3	99.5	0.5	0.43	塩化金酸*2	SD－3*5
Em－4	10	90	0.67		SD－1*3
Em－5	30	70	0.46	チオ硫酸	SD－2*4
Em－6	30	70	0.43	ナトリウム*1	SD－3*5

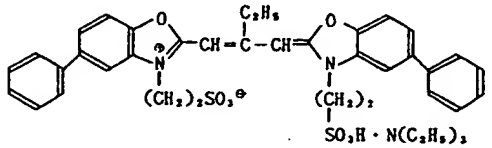
*1　ハロゲン化銀1モル当り2mg添加
*2　ハロゲン化銀1モル当り5×10^{-5}モル添加
*3　ハロゲン化銀1モル当り0.9ミリモル添加
*4　ハロゲン化銀1モル当り0.7ミリモル添加
*5　ハロゲン化銀1モル当り0.2ミリモル添加

それぞれのハロゲン化銀乳剤は化学増感終了後に乳剤安定剤として下記に示すSTB－1をハロゲン化銀1モル当り、5×10^{-3}モル添加した。

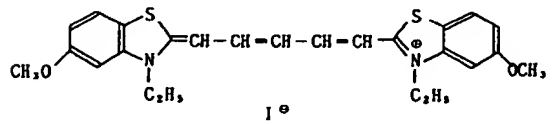
〔SD－1〕



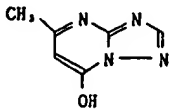
〔SD－2〕



〔SD－3〕



〔STB－1〕



（ハロゲン化銀カラー写真感光材料試料の作製）

次いで以下の層１～７を両面をポリエチレンで被覆した紙支持体上に順次塗設（同時塗布）し、ハロゲン化銀カラー写真感光材料16～22を作製した。（なお、以下の実施例において、添加量は感光材料１m²当りの量で示す。）

層１…ゼラチン（1.2g）と0.29g（銀換算、以下同じ）の青感光性ハロゲン化銀乳剤（Em－１）そして0.75gのイエローカプラー（Y－１）、0.3gの光安定剤ST－１及び0.015gの２，５-ジオクチルハイドロキノン（HQ－１）を溶解した0.3gのジノニルフタレート（DNP）を含有している層。

層２…ゼラチン（0.9g）と0.04gのHQ－１を溶解した0.2gのDOP(ジオクチルフタレート）を含有している層。

層３…ゼラチン（1.4g）と0.2gの緑感光性ハロゲン化銀乳剤（Em－２）と0.50のマゼンタカプラー（M－１）、0.25gの光安定剤（ST－２）及び0.01gのHQ－１を溶解した0.3gのDOPと6mgの下記フィルター染料（AI－１）を含有している層。

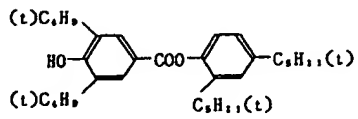
層４…ゼラチン（1.2g）と下記の0.6gの紫外線吸収剤（UV－１）及び0.05gのHQ－１を溶解した0.3gのDNPを含有している層。

層５…ゼラチン（1.4g）と0.20gの赤感光性ハロゲン化銀乳剤（Em－３）及び0.9ミリモルの表－３に示すシアンカプラーと0.01gのHQ－１を溶解した0.3gのDOPを含有している層。

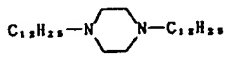
層６…ゼラチン（1.1g）と0.2gのUV－１を溶解した0.2gのDOPおよび５mgの下記フィルター染料（AI－２）を含有している層。

層７…ゼラチン（1.0g）と0.05gの２，４-ジクロロ-６-ヒドロキシトリアジンナトリウムを含有している層。

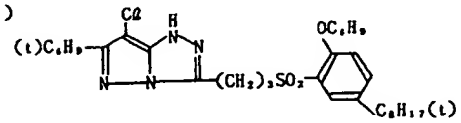
(ST－１)



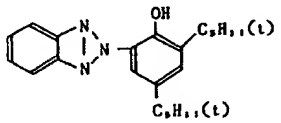
(ST－２)



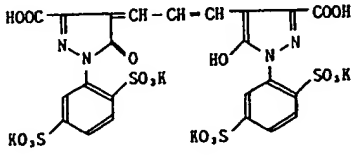
(M－１)



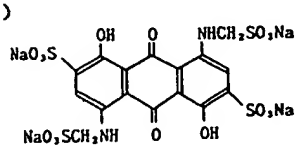
(UV－１)



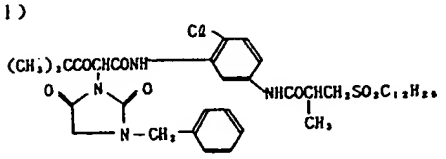
(AI－１)



(AI－２)



(Y－１)



得られた試料を感光計 KS－７型（コニカ株式会社製）を使用して、ウェッジ露光後、以下の発色現像処理工程に従って処理した後、光学濃度計（コニカ株式会社製PDA－65型）を用いて赤感光性乳剤層の感度（カブリ＋0.1の濃度を与える露光量の逆数）および最高濃度（Dmax）を測定した。

また、得られた試料を85℃，60%の相対湿度に20日間保存し、初濃度 1.0 における色素画像の残存率（%）を求めることにより暗褪色性を評価した。結果を表－３に示す。

〔処理工程〕

	温　度	時　間
発色現像	34.7±0.3℃	45秒
漂白定着	34.7±0.5℃	50秒
安定化	30～34℃	90秒
乾　燥	60～80℃	60秒

〔発色現像液〕

純水	800mℓ
トリエタノールアミン	8g
N,N-ジエチルヒドロキシルアミン	5g
塩化カリウム	2g
N-エチル-N-β-メタンスルホンアミドエチル-3-メチル-4-アミノアニリン硫酸塩	5g
テトラポリ燐酸ナトリウム	2g
炭酸カリウム	30g
亜硫酸カリウム	0.2g
蛍光増白剤（4,4'-ジアミノスチルベンジスルホン酸誘導体）	1g

純水を加えて全量を1ℓとし、pH 10.2に調整する。

〔漂白定着液〕

エチレンジアミン四酢酸第2鉄アンモニウム2水塩	60g
エチレンジアミン四酢酸	3g
チオ硫酸アンモニウム（70%溶液）	100mℓ
亜硫酸アンモニウム（40%溶液）	27.5mℓ

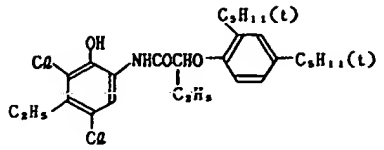
水を加えて全量を1ℓとし、炭酸カリウムまたは氷酢酸でpH 5.7に調整する。

〔安定化液〕

5-クロロ-2-メチル-4-イソチアゾリン-3-オン	1g
1-ヒドロキシエチリデン-1,1-ジホスホン酸	2g

水を加えて1ℓとし、硫酸または水酸化カリウムでpHを7.0に調整する。

比較カプラー（C－3）



以下余白

表－3

試料 No.	カプラー	感度*	Dmax	色画像残存率
16(比較)	C－2	100	2.55	80%
17(〃)	C－3	97	2.55	92
18(本発明)	例示10	115	2.63	94
19(〃)	〃 18	110	2.66	95
20(〃)	〃 35	112	2.65	94
21(〃)	〃 37	110	2.61	96
22(〃)	〃 38	108	2.62	95

*感度は試料16の感度を100とした時の相対値で示す。

表－3の如く本発明のシアンカプラーを用いた時、高い最高濃度、感度が得られ、また得られたシアン画像の耐熱性も良好であることがわかる。

また、これらの試料16,17,20,21,22に、コニカカラーGX-100にてカラーチェッカー、（マクベス社製）を撮影したカラーネガとニュートラルを合わせた条件下でプリントしたサンプルの色再現性を目視にて評価した。

試料16,17に比べ、本発明の試料である20,21,22はいずれも青,シアンの識別、緑色、赤～マゼンタの色再現性が非常に改善されていた。

実施例－3

実施例－2で作製したハロゲン化銀カラー写真感光材料の層1の青感光性ハロゲン化銀乳剤を表－2のEm－4に、層3の緑感光性ハロゲン化銀乳剤をのEm－5に、層5の赤感光性ハロゲン化銀乳剤をEm－6に、それぞれ代えた以外はすべて実施例－2と同様にしてハロゲン化銀カラー写真感光材料試料23～29を作製した。

得られた試料を感光計KS－7型（コニカ株式会社製）を使用してウェッジ露光後、以下の発色現像処理工程に従って処理した後、実施例－2と同様な測定を行った。

結果を表－4に示す。

〔処理工程〕

発色現像	3分30秒	温度33℃
漂白定着	1分30秒	温度33℃
水洗	3分	温度33℃

発色現像液処方

N-エチル-N-β-メタンスルホンアミドエチル-3-メチル-4-アミノアニリン硫酸塩	4.9g
ヒドロキシルアミン硫酸塩	2.0g
炭酸カリウム	25.0g
臭化カリウム	0.6g
無水亜硫酸ナトリウム	2.0g
ベンジルアルコール	13mℓ
ポリエチレングリコール (平均重合度 400)	3.0mℓ

水を加えて1ℓとし、水酸化ナトリウムでpH 10.0に調整する。

漂白定着液処方

エチレンジアミン四酢酸鉄ナトリウム塩	6.0g
チオ硫酸アンモニウム	100g
重亜硫酸ナトリウム	10g
メタ重亜硫酸ナトリウム	3g

水を加えて1ℓとし、アンモニア水でpH 7.0に調整する。

表－4

試料 No.	カプラー	感度*	D max	色画像残存率
23(比 較)	C－2	100	2.50	81%
24(〃)	C－3	101	2.52	91
25(本発明)	例示10	118	2.68	93
26(〃)	〃 18	113	2.66	92
27(〃)	〃 35	110	2.70	94
28(〃)	〃 37	115	2.71	95
29(〃)	〃 38	117	2.68	95

*感度は実施例－2と同様に試料23の感度を100とした時の相対値で示す。

以上の如く、実施例－2と同様な結果が得られた。

実施例－4

以下の乳剤を調製した。

乳剤Sの調製

2.0%不活性ゼラチン液750mℓを50℃に保ち、撹拌しながら下記A1液とB液を同時に加え、3分間かけて注入した。25分間熟成後、沈澱水洗法により過剰塩を除去した後、再分散させC1液とD1液を加えた。10分後、再度過剰の水溶性塩を除去しゼラチンを少量加えてハロゲン化銀粒子を分散させた。

乳剤Lの調製

1.5%不活性ゼラチン液750mℓを60℃に保ち、撹拌しながらA2液とB液を同時に加え、15分かけて注入した。40分間熟成後、沈澱水洗法により過剰塩を除去した後、再分散させハイポ10mgを加えた後C2液とD2液を加えた。10分後、再度過剰の水溶性塩を除去しゼラチンを少量加えてハロゲン化銀粒子を分散させた。

乳剤Mの調製

2.0%不活性ゼラチン液750mℓを50℃に保ち、撹拌しながら下記A3液とB液を同時に加え、5分間かけて注入した。25分間熟成後、沈澱水洗法により過剰塩を除去した後、再分散させC1液とD2液を加えた。10分後、再度過剰の水溶性塩を除去し、ゼラチンを少量加えてハロゲン化銀粒子を分散させた。

A1液	純水	2000 mℓ
	NaCℓ	35 g
	NH_4Br	109.6g
	KI	0.8g
A2液	純水	1000 mℓ
	NaCℓ	26.3g
	NH_4Br	109.6g
	KI	0.8g
A3液	純水	1000 mℓ
	NaCℓ	33.8g
	KBr	12 g
B液	純水	1200 mℓ
	$AgNO_3$	170 g
C1液	純水	1000 mℓ
	NaCℓ	60 g
	NH_4Br	6.9g
C2液	純水	1000 mℓ
	NaCℓ	31.6g
D1液	純水	1000 mℓ
	$AgNO_3$	70 g

D 2 液
- 純水 1000 mℓ
- $AgNO_3$ 80 g

この3種の乳剤に下記のように、増感色素、カプラー等を加えて支持体上に塗布し、多層カラー感光材料を作成した。

赤感光乳剤層(第1層)

乳剤S及び乳剤Mに対し、それぞれ増感色素[PD-3]、[PD-4]、安定剤[STB-1]、[STB-2]、界面活性剤[S-2]、更にジブチルフタレート、酢酸エチル、界面活性剤[S-2]、2,5-ジオクチルハイドロキノン及びシアンカプラー[C-2]、[C-4]を含むプロテクト分散されたカプラー液を加えた。

ゼラチンを加え、それぞれの乳剤を混合してガンマ値が、1.5となるように塗布する。

第1中間層(第2層)

ジオクチルフタレート2,5-ジオクチルハイドロキノン、紫外線吸収剤チヌビン328(チバガイギー社製)、界面活性剤[S-1]を含むプロテクト分散された液を含むゼラチン液を用意しチヌビン塗布量0.15/m²となるように塗布。

緑感光乳剤層(第3層)

乳剤Sと乳剤Mに対し増感色素[PD-2]、安定剤[STB-1]、[STB-2]、界面活性剤[S-2]、更にジブチルフタレート、酢酸エチル、2,5-ジオクチルハイドロキノン、界面活性剤[S-1]、マゼンタカプラー[M-2]を含むプロテクト分散されたカプラー液を加えた。

ゼラチンを加え、更に硬膜剤[H-1]を加えて、できた乳剤のガンマ値が、1.5になるように塗布。

第2中間層(第4層)

第1中間層と同一処方でチヌビン328塗布量を0.2g/m²となるように塗布。

イエローフィルター層(第5層)

アルカリ性弱還元剤下で酸化して作られた(中和後ヌードル水洗法により弱還元剤を除去)イエローコロイド銀およびジオクチルフタレート、酢酸エチル、界面活性剤[S-1]、2,5-ジオクチルハイドロキノン液、界面活性剤[S-2]および硬膜剤[H-1]を加えてコロイド銀塗布量0.15g/m²となるように塗布。

第3中間層(第6層)

第1中間層と同じ。

青感光乳剤層(第7層)

乳剤L、乳剤S及び乳剤Mに対し、それぞれ増感色素[PD-1]、安定剤[STB-1]、[STB-3]、界面活性剤[S-2]、更にジブチルフタレート、酢酸エチル、2,5-ジオクチルハイドロキノン、界面活性剤[S-1]及びイエローカプラー[Y-2]を含むプロテクト分散されたカプラー液を加えた。

ゼラチンを加え、更に硬膜剤[H-1]を加えて、できた乳剤をガンマ値が1.5になるよう混合して塗布する。

第3中間層(第8層)

第1中間層と同じ処方でチヌビン328塗布量0.35g/m²となるように塗布。

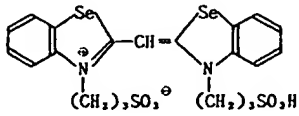
保護層(第9層)

コロイダルシリカ、塗布助剤[S-2]、硬膜剤[H-2]、[H-3]を含むゼラチン液を用いゼラチン塗布量1.0g/m²となるよう塗布。

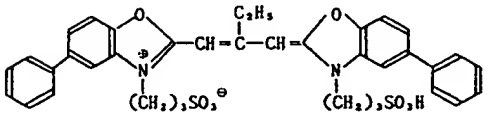
表面処理のされたポリエチレンラミネート紙上に第1層から第9層迄を同時塗布方式により塗布し乾燥した(試料30)。

更に赤感光性層(第1層)のシアンカプラーを表-5に示すように等モルの本発明のカプラーに変えた以外は同様にして試料31～33を作製した。

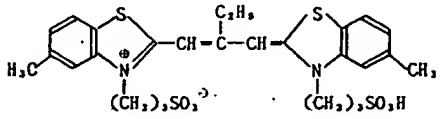
〔PD-1〕



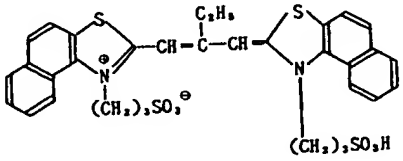
〔PD-2〕



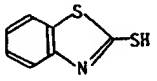
〔PD-3〕



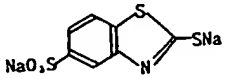
〔PD－4〕



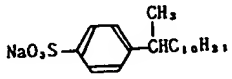
〔STB－2〕



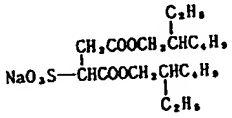
〔STB－3〕



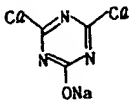
〔S－1〕



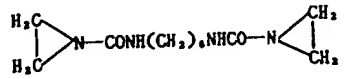
〔S－2〕



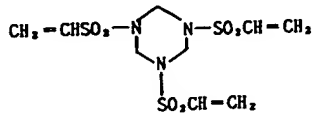
〔H－1〕



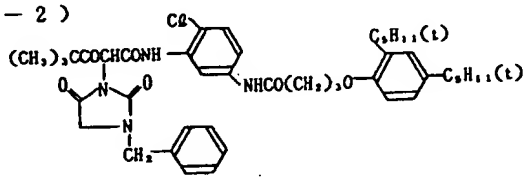
〔H－2〕



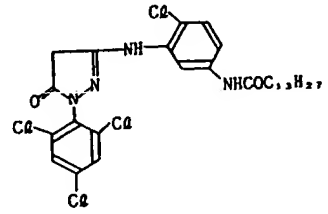
〔H－3〕



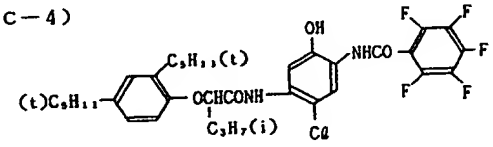
〔Y－2〕



〔M－2〕



〔C－4〕



上記のように作製した感光材料試料30～33に対して白色ウェッジ露光を施し、以下の工程に従って現像処理を施し、ニュートラルのポジウェッジを得た。

このシアン画像のDmaxを測定すると共に、この画像を85℃、60%の相対湿度下に20日保存した時の濃度1.0におけるシアン画像の残存率を測定した。結果を表－5に示す。

表中、各感光層の乳剤組成は重量部を表す。

処理工程（処理温度と処理時間）

[1]浸漬（発色現像液）	38℃	8秒
[2]カブリ露光	— 1ルックスで10秒	
[3]発色現像	38℃	2分
[4]漂白定着	35℃	60秒
[5]安定化処理	25～30℃	1分30秒
[6]乾燥	75～80℃	1分

処理液組成

（発色現像液）

ベンジルアルコール	10mℓ
エチレングリコール	15mℓ
亜硫酸カリウム	2.0g
臭化カリウム	1.5g
塩化ナトリウム	0.2g
炭酸カリウム	30.0g
ヒドロキシルアミン硫酸塩	3.0g
ポリ燐酸（TPPS）	2.5g
3-メチル-4-アミノ-N-エチル-N-（β-メタンスルホンアミドエチル）アニリン硫酸塩	5.5g
蛍光増白剤（4,4'-ジアミノスチルベンジスルホン酸誘導体）	1.0g
水酸化カリウム	2.0g

水を加えて全量1ℓとし、pH 10.20に調整する。

（漂白定着液）

エチレンジアミン四酢酸第2鉄アンモニウム2水塩	60g
エチレンジアミン四酢酸	3g
チオ硫酸アンモニウム（70%溶液）	100mℓ
亜硫酸アンモニウム（40%溶液）	27.5mℓ

水を加えて全量を1ℓとし、炭酸カリウムまた

は水酢酸でpH 7.1に調整する。

(安定化液)

5-クロロ-2-メチル-4-イソチアゾリン-3-オン	1.0g
エチレングリコール	10g
1-ヒドロキシエチリデン-1,1'-ジホスホン酸	2.5g
塩化ビスマス	0.2g
塩化マグネシウム	0.1g
水酸化アンモニウム(28%水溶液)	2.0g
ニトリロ三酢酸ナトリウム	1.0g

水を加えて全量を1lとし、水酸化アンモニウムまたは硫酸でpH 7.0に調整する。

なお安定化処理は2槽構成の向流方式にした。

表－5

試料No.	カプラー	Dmax	残存率
30(比　較)	C－2 C－4	2.24	88%
31(本発明)	例示10	2.41	94
32(　〃　)	〃 18	2.35	95
33(　〃　)	〃 35	2.30	95

以上の如く、本発明カプラーは耐熱性が良好なことがわかる。また、この感光材料をコニカカラー7に裁断し、1×20cmの長方形スリットから5cm/secの一定速度で走査露光することにより、カラーチェッカー(マクベス社製)をコピーしたところ、試料31～33については、比較試料30よりもシアンとブルーの色相差がはっきりとわかり、緑および赤～マゼンタの色再現が改良されていた。

実施例－5

背面帯電防止処理をした後、無水マレイン酸と酢酸ビニル共重合体で下引き加工したトリアセチルセルロースフィルム支持体上に下記組成の各層を支持体側より順次塗布して多層カラー感光材料試料34を作製した。添加量は特に記載がない限りハロゲン化銀1モル当りのものを示す。

＜背面帯電防止処理＞

背面1層：	ステアリン酸	20mg/m²
	ジアセチルセルロース	10mg/m²
	アルミナゾル	1g/m²
背面2層：	ジアセチルセルロース	50mg/m²
	ステアリン酸	10mg/m²
	シリカマット剤(平均粒径3μm)	50mg/m²

＜支持体上＞

第1層：ハレーション防止層

紫外線吸収剤UV－2 0.3g/m²，紫外線吸収剤UV-3 0.4g/m²，黒色コロイド銀 0.24g/m²，ゼラチン 2.7g/m²

第2層：中間層

2,5-ジ-t-オクチルハイドロキノン 0.1g/m²，ゼラチン 1.0g/m²

第3層：低感度赤感性ハロゲン化銀乳剤層

平均粒径0.35μm AgI2.5モル%を含むAgBrIからなる単分散乳剤(乳剤I)…銀量0.5g/m²

増感色素－1…7.6×10⁻⁵モル

カプラーC-5…0.1モル，ゼラチン 0.9g/m²

第4層：高感度赤感性ハロゲン化銀乳剤層

平均粒径0.75μm AgI2.5モル%を含むAgBrIからなる単分散乳剤(乳剤II)…銀量 0.8g/m²

増感色素-1…3.2×10⁻⁵モル

カプラーC-5…0.2モル，ゼラチン 1.75g/m²

第5層：中間層

2,5-ジ-t-オクチルハイドロキノン 0.1g/m²

ゼラチン 0.9g/m²

第6層：低感度緑感性ハロゲン化銀乳剤層

乳剤I…銀量 1.0g/m²

増感色素-2…6.6×10⁻⁵モル

増感色素-3…0.6×10⁻⁵モル

カプラーM-3…0.05モル，ゼラチン 0.8g/m²

第7層：高感度緑感性ハロゲン化銀乳剤層

乳剤II…銀量 1.0g/m²

増感色素-2…2.76×10⁻⁵モル

増感色素-3…0.23×10⁻⁵モル

カプラーM-3…0.15モル，ゼラチン 1.5g/m²

第8層：中間層

第5層と同じ

第9層：イエローフィルター層

黄色コロイド銀 0.1g/m²，ゼラチン 0.9g/m²

2,5-ジ-t-オクチルハイドロキノン 0.1g/m²

第10層：低感度青感性ハロゲン化銀乳剤層

平均粒径0.6μm AgI2.5モル％を含むAgBrIからなる単分散乳剤（乳剤Ⅲ）…銀量　0.4g/m²

分光増感色素－4 …2.65×10⁻⁴モル

カプラーY－3 …0.3モル，ゼラチン　1.3g/m²

第11層：高感度青感性ハロゲン化銀乳剤層

平均粒径　1.0μm AgI2.5モル％を含むAgBrIからなる単分散乳剤（乳剤Ⅳ）…銀量　0.8g/m²

分光増感色素－4 …1.59×10⁻⁴モル，

カプラーY－3 …0.3モル，ゼラチン　2.1g/m²

第12層：第1保護層

UV－2　0.3g/m²，UV－3　0.4g/m²，

ゼラチン　1.2g/m²，2.5-ジ-t-オクチルハイドロキノン　0.1g/m²

第13層：第2保護層

平均粒径　0.06μm AgI 1モル％を含むAgBrIからなる非感光性微粒子ハロゲン化銀乳剤…銀量　0.3g/m²，ポリエチルメタクリレート粒子（直径1.5μm），ゼラチン　0.7g/m²および界面活性剤S－3

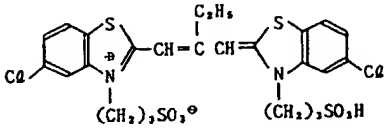
尚、各層には上記組成物の他にH－1や界面活性剤を添加した。また、カプラーの溶媒としてトリクレジルホスフェートを用いた。

なお、乳剤はすべて単分散性の8面体乳剤であり、0.095μmあるいは0.25μmの種乳剤（平均沃化銀含有率2モル％）を45℃にてアンモニア存在下pAg、pHをコントロールしたダブルジェット法により成長させたものである。コア、中間層およびシェルの沃化銀含有率は添加するハロゲン化銀の組成を変更することにより行った。

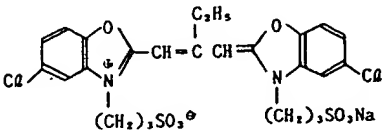
コア／シェル型ハロゲン化銀乳剤の成長には特開昭59-52238号、同60-138538号、同58-49938号、同60-122935号記載の方法を用いた。

（試料を作るのに使用した化合物）

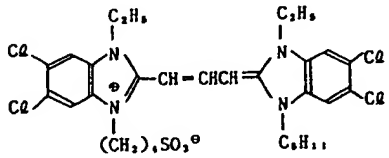
増感色素－1



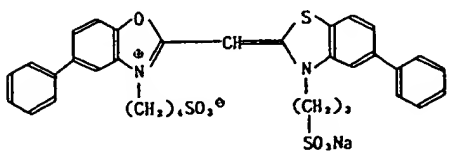
増感色素－2



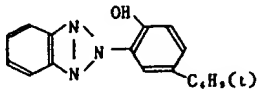
増感色素－3



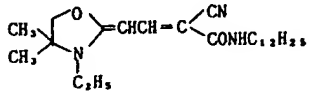
増感色素－4



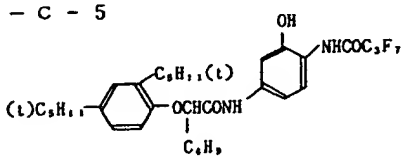
紫外線吸収剤UV－2



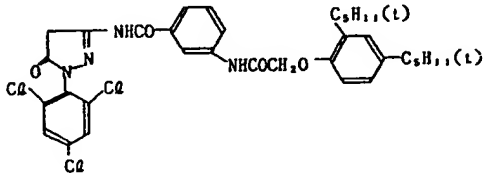
紫外線吸収剤UV－3



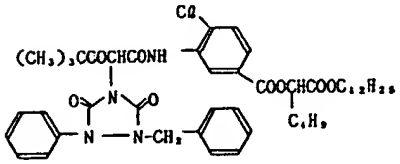
カプラーC－5



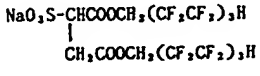
カプラーM－3



カプラーY－3



界面活性剤－1



次に試料34の第3層及び第4層のシアンカプラーC-5を本発明のカプラー1，4，9，34に代え、第3層及び第4層の全体の付量を30%減らしたものを作製し、それぞれ試料35.36.37及び38とした。

試料34～38をウエッジ露光し、以下の反転処理を施した後、赤色光でシアン画像のDmaxを測定した。また、試料34～38を実際に使用してカラーチェッカー(マクベス社製)を撮影し、反転処理を施した後に目視にて色再現性を比較した。

結果を表－6に示す。

処理工程	処理時間	処理温度
第1現像	6′	38℃（±0.3）
水洗	2′	38℃（±0.3）
反転	2′	38℃（±0.3）
発色現像	6′	38℃（±0.3）
調整	2′	38℃（±0.3）
漂白	6′	38℃（±0.3）
定着	4′	38℃（±0.3）
水洗	4′	38℃（±0.3）
安定	1′	常温
乾燥		

第1現像液

水	700mℓ
テトラポリ燐酸ナトリウム	2g
亜硫酸ナトリウム	20g
ハイドロキノン・モノスルホネート	30g
炭酸ナトリウム（1水塩）	30g
1－フェニル－4－メチル－4－ヒドロキシメチル－3－ピラゾリドン	2g
臭化カリウム	2.5g
チオシアン酸カリウム	1.2g
沃化カリウム（0.1%溶液）	2mℓ
水を加えて	1000mℓ

反転液

水	700mℓ
ニトリロトリメチレンホスホン酸・6ナトリウム塩	3g
塩化第1錫（2水塩）	1g
p－アミノフェノール	0.1g
水酸化ナトリウム	8g
氷酢酸	15mℓ
水を加えて	1000mℓ

発色現像液

テトラポリ燐酸ナトリウム	2g
亜硫酸ナトリウム	7g
第3燐酸ナトリウム（2水塩）	36g
臭化カリウム	1g
沃化カリウム(0.1%溶液)	90mℓ
水酸化ナトリウム	3g
シトラジン酸	1.5g
N－メチル－N－（β－メタンスルホンアミドエチル）－3－メチル－4－アミノアニリン硫酸塩	11g
エチレンジアミン	3g
水を加えて	1000mℓ

調整液

水	700mℓ
亜硫酸ナトリウム	12g
エチレンジアミン四酢酸ナトリウム（2水塩）	8g
チオグリセリン	0.4mℓ
氷酢酸	3mℓ
水を加えて	1000mℓ

漂白液

エチレンジアミン四酢酸ナトリウム（2水塩）	2.0g
エチレンジアミン四酢酸鉄（Ⅲ）アンモニウム（2水塩）	120.0g
臭化アンモニウム	100.0g
水を加えて	1000mℓ

定着液

水	800mℓ
チオ硫酸アンモニウム	80.0g
亜硫酸ナトリウム	5.0g
重亜硫酸ナトリウム	5.0g
水を加えて	1000mℓ

安定液

水	800mℓ
ホルマリン（37重量%）	5.0mℓ
コニダックス(コニカ株式会社製)	5.0mℓ

水を加えて　　1000mℓ

表－6

試料No	カプラー	Dmax(赤)
34(比　較)	C－5	3.10
35(本発明)	例示　1	3.26
36(　〃　)	〃　4	3.25
37(　〃　)	〃　9	3.23
38(　〃　)	〃　34	3.24

この結果からわかる様に、本発明試料は層3、層4の付量が減っているにもかかわらず、本発明カプラーのモル吸光係数が高いために比較試料と同等以上のDmaxが出ていることがわかる。

また、実際にカラーチェッカーを撮影したものを目視で評価した限りでは、比較試料34に較べ本発明の試料35～38は、本発明カプラーの特徴であるシアンと青の識別が良く、緑および赤の色再現が改善されていた。

実施例－6

特願昭61-31330号に記載された方法により調製されたハロゲン化銀乳剤、即ち粒子内部の高沃度殻から外側に向って沃度含量が低くなるような多殻構造を持つコア／シェル沃臭化銀乳剤に、常法に従い化学増感を施し、下記の添加剤を加えた各層塗布液を、トリアセチルセルロースフィルム支持体上に順次支持体側から各層を塗設して、13層から成るカラー感光材料試料39を作製した。

第1層；ハレーション防止層

黒色コロイド銀を含むゼラチン層。

ゼラチン　2.2g/m²

第2層；中間層

2,5-ジ-t-オクチルハイドロキノンの乳化分散物を含むゼラチン層。

ゼラチン　1.2g/m²

第3層；低感度赤感性ハロゲン化銀乳剤層

平均粒径　0.38μm、平均沃度含量7.84%（重量比）の八面体型沃臭化銀からなる単分散乳剤(乳剤Ⅰ)…

銀塗布量1.8g／m²

増感色素－5…

銀1モルに対して6×10⁻⁵モル

増感色素－6…

銀1モルに対して1.0×10⁻⁵モル

増感色素－7

銀1モルに対して1.0×10⁻⁵モル

シアンカプラー(C－6)…

銀1モルに対して0.02モル

カラードシアンカプラー(CC－1)…

銀1モルに対して0.0015モル

DIR化合物(D－2)…

銀1モルに対して0.001モル

ゼラチン　1.0g/m²

第5層；中間層

第2層と同じ、ゼラチン層。

ゼラチン　1.0g/m²

第6層；低感度緑感性ハロゲン化銀乳剤層

乳　剤－Ⅰ…塗布銀量　1.5g／m²

増感色素－8…

銀1モルに対して2.5×10⁻⁵モル

増感色素－9…

銀1モルに対して1.2×10⁻⁵モル

増感色素－10…

銀1モルに対して1.0×10⁻⁵モル

増感色素－7…

銀1モルに対して1.0×10⁻⁵モル

シアンカプラー(C－6)…

銀1モルに対して0.06モル

カラードシアンカプラー(CC－1)…

銀1モルに対して0.003モル

DIR化合物(D－1)…

銀1モルに対して0.0015モル

DIR化合物(D－2)…

銀1モルに対して0.002モル

ゼラチン1.4g/m²

第4層；高感度赤感性ハロゲン化銀乳剤層

平均粒径　0.65μm,平均沃度含量　7.37%（重量比）の八面体型沃臭化銀からなる単分散乳剤（乳剤Ⅱ）…

…銀塗布量1.3g／m²

増感色素－5…

銀1モルに対して3×10⁻⁵モル

増感色素－6…

銀1モルに対して1.0×10⁻⁵モル

マゼンタカプラー(M－4)…

銀1モルに対して0.05モル

カラードマゼンタカプラー(CM－1)…

銀1モルに対して0.009モル

DIR化合物(D－1)…

銀1モルに対して0.0010モル

DIR化合物(D－3)…

銀1モルに対して0.0030モル

ゼラチン 2.0g/m²

第7層：高感度緑感性ハロゲン化銀乳剤層

乳剤－II…塗布量銀1.4g／m²

増感色素－8…

銀1モルに対して1.5×10⁻⁵モル

増感色素－9…

銀1モルに対して1.0×10⁻⁵モル

増感色素－10…

銀1モルに対して7.0×10⁻⁵モル

マゼンタカプラー(M－4)…

銀1モルに対して0.020モル

ゼラチン 1.9g/m²

第11層：高感度青感性乳剤層

単分散乳剤(乳剤II)…

銀塗布量0.5g／m²

増感色素－11…

銀1モルに対して1.0×10⁻⁵モル

イエローカプラー(Y－4)…

銀1モルに対して0.08モル

DIR化合物(D－2)

銀1モルに対して0.0015モル

ゼラチン 1.6g/m²

第12層：第1保護層

沃臭化銀(AgI 1モル%平均粒径0.07μm)

銀塗布量0.5g／m²

紫外線吸収剤 UV－2，UV－3を含むゼラチン層。

ゼラチン 1.2g/m²

第13層：第2保護層(Pro－2)

ポリメチルメタクリレート粒子

(直径1.5μm)

カラードマゼンタカプラー(CM－1)…

銀1モルに対して0.002モル

DIR化合物(D－3)…

銀1モルに対して0.0010モル

ゼラチン 1.8g/m²

第8層 中間層

第2層と同じ、ゼラチン層

ゼラチン 1.0g/m²

第9層：イエローフィルター層

黄色コロイド銀と2,5-ジ-t-オクチルハイドロキノンの乳化分散物とを含むゼラチン層。

ゼラチン 1.5g/m²

第10層：低感度青感性ハロゲン化銀乳剤層

単分散乳剤(乳剤I)…

銀塗布量0.9g／m²

増感色素－11…

銀1モルに対して1.3×10⁻⁵モル

イエローカプラー(Y－4)…

銀1モルに対して0.29モル

エチルメタクリレート：メチルメタクリレート：メタクリル酸の共重合体粒子

(平均粒径 2.5μm)

ポリジメチルシロキサン 5mg/m²

$C_8F_{17}SO_2NCH_2COONa$ (C₂H₅) 10mg/m²

及びホルマリンスカベンジャー

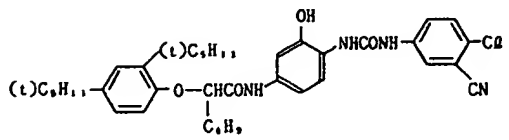
(HS－1)を含むゼラチン層

ゼラチン1.2g/m²

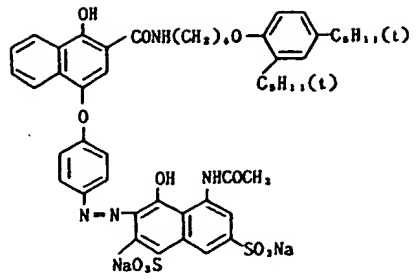
尚各層には上記組成物の他に、ゼラチン硬化剤(H－1)、界面活性剤を添加した。

(添加剤)

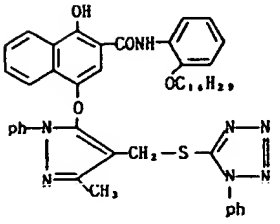
C－6



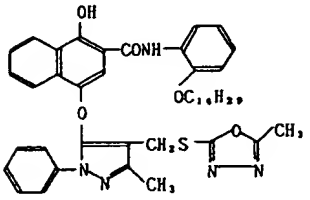
C C － 1



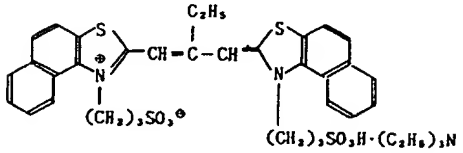
D － 1



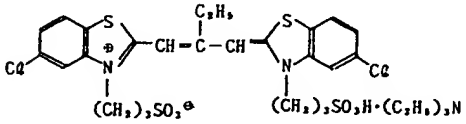
D － 2



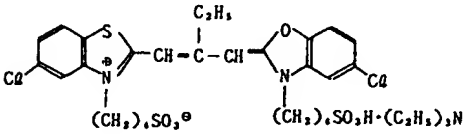
増感色素－5



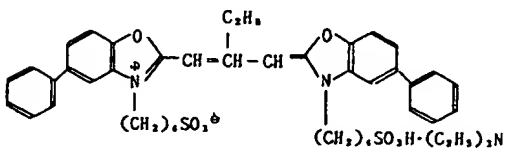
増感色素－6



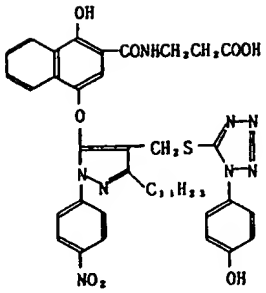
増感色素－7



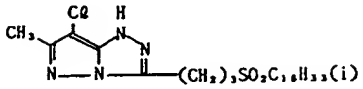
増感色素－8



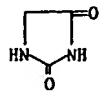
D － 3



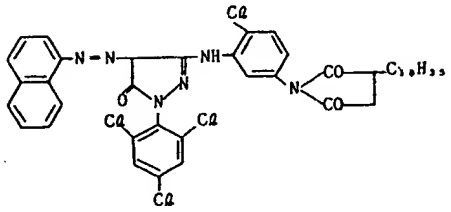
M － 4



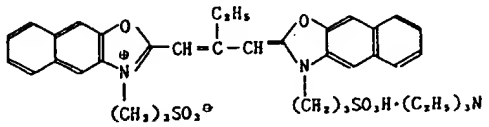
H S － 1



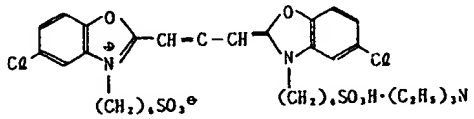
C M － 1



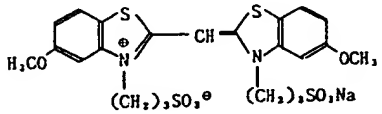
増感色素－9



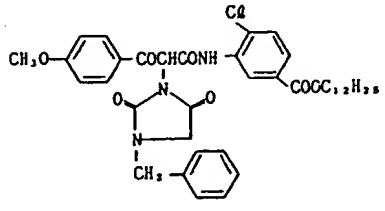
増感色素－10



増感色素－11



Y － 4



また、第3層および第4層のシアンカプラーC－6を表－7に示す　に比較カプラーの0.6モル倍の本発明のカプラー4,6,9,16,に代えたものを試料40,41,42,43とした。

この様にして作製した各試料を白色光でウェッジ露光し、下記現像処理を行い、パンクロ層の感度とカブリを求めた。感度はカブリ＋0.5の濃度を与える露光量の逆数で表し、試料39の感度を100とした時の相対値で示した。

又、シアン画像について85℃、60%相対湿度下に20日間保存した時の濃度1.0における劣化後の残存率を測定した。結果を併せて表－7に示す。

処理工程(38℃)

発色現像	3分15秒
漂　白	6分30秒
水　洗	3分15秒
定　着	6分30秒
水　洗	3分15秒
安定化	1分30秒
乾　燥	

[発色現像液]

4-アミノ-3-メチル-N-エチル-N-(β-ヒドロキシエチル)アニリン・硫酸塩	4.75g
無水亜硫酸ナトリウム	4.25g
ヒドロキシルアミン・1/2硫酸塩	2.0g
無水炭酸カリウム	37.5g
臭化ナトリウム	1.3g
ニトリロ三酢酸・3ナトリウム塩(1水塩)	2.5g
水酸化カリウム	1.0g

水を加えて1ℓとする。

[漂白液]

エチレンジアミン四酢酸鉄アンモニウム塩	100.0g
エチレンジアミン四酢酸2アンモニウム塩	10.0g
臭化アンモニウム	150.0g
氷酢酸	10.0mℓ

水を加えて1ℓとし、アンモニア水を用いてpH＝6.0に調整する。

[定着液]

チオ硫酸アンモニウム	175.0g
無水亜硫酸ナトリウム	8.5g
メタ亜硫酸ナトリウム	2.3g

水を加えて1ℓとし、酢酸を用いてpH＝6.0に調整する。

[安定液]

ホルマリン(37%水溶液)	1.5mℓ
コニダックス(コニカ株式会社製)	7.5mℓ

水を加えて1ℓとする。

表－7

試料No.	カプラー	感　度	残存率
39(比　較)	C－6	100	80%
40(本発明)	例示4	118	91
41(　〃　)	〃　6	120	94
42(　〃　)	〃　9	120	93
43(　〃　)	〃　16	128	90

各処理工程において使用した処理液組成は下記の通りである。

これらの結果から、本発明カプラーを用いた試料は比較試料に比べ少ないカプラー量にもかかわらず、高い感度を与え、また耐熱性も優れていることがわかる。

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(54) Artificial carrier particles and method for preparation thereof.

(57) An artificial carrier particle comprising an anionic polymer and a synthetic polyamino acid having at least one carboxylic group and at least one amino group in its side chain, the complex being insolubilized by an aldehyde crosslinking agent. The artificial carrier particle is useful in immunoassays, in particular, particle immunoassays. The artificial carrier particles are obtained by preparing an aqueous solution containing an anionic polymer and a synthetic polyamino acid comprising at least one free carboxyl group and at least one free amino group in its side chain, adjusting the pH of the solution to 3.5 to 9.5 at room temperature or at progressively increasing temperature under stirring to form a solution of particles of a desired particle size, and insolubilizing the particles by an aldehyde crosslinking agent.

EP 0 363 921 A2

Artificial Carrier Particles and Method for Preparation thereof

The present invention relates to novel artificial particles useful as carriers for immunoassays, in particular, particle immunoassays, to the preparation thereof and to immunoassay reagents comprising the particles.

The method for particle immunoassays is based on the steps of using particles of appropriate size as carriers, sensitizing (adsorbing or binding) antigens or antibodies onto the carriers and observing the agglutination of the thus sensitized carriers caused by antibodies or antigens corresponding thereto.

Examples of the most popular carriers conventionally used in particle immunoassays include erythrocytes of various animals such as sheep and chicken, and artificial synthetic polymer particles such as polystyrene latex particles. Although the former method in which the erythrocytes of animals are used as the carriers has been known as one utilizing the so-called hemagglutination, this method has advantages in that it can be applied to many kinds of antigens and antibodies and, for example, the immunoassay can be finished in a short time (1 to 2 hours) by employing the microtiter method. However, there are disadvantages in that the erythrocytes per se have inherent antigenicity so that non-specific agglutination arises easily, and that differences in the properties of the erythrocytes depending on the individual, season and the like are very large and the erythrocytes cannot be obtained in a uniform quality since they are collected from living animals. Furthermore, although there is an advantage in that the size of the erythrocytes is constant for a given kind of animal, this causes a disadvantage in that there cannot be obtained particles having a desired size in accordance with the purpose of use.

On the other hand, synthetic polymer particles such as polystyrene latex generally have a particle diameter of about 0.1~ 1 μm and the particles are particular useful as carriers for an agglutination reaction. The particles have advantages in that the particles per se have no antigenicity and can be constantly obtained in a uniform quality and in a large amount. However, in the case where the microtiter method is used in order to obtain a high sensitivity and to conduct a quantitative analysis, the carriers consisting of the conventional synthetic polymer particles have a problem in that a long time period is required for sedimention, compared with the case of using erythrocytes as carriers and, therefore, the assay cannot be finished rapidly. In addition, there is a possibility of natural agglutination, i.e., non-specific agglutination, occurring in the natural pH region preferable for an immunoreaction medium.

Although it is known that natual inorganic particles such as kaolin and carbon powder are useful as the carriers mentioned above, there particles have disadvantages in that the antibodies or antigens are difficult to sensitize to a high degree and cannot easily be selected in a constant particle range, and therefore, the particles can only be used in an extremly limited field.

Furthermore, there has recently been developed an artificial carrier comprising gelatin, water soluble polysaccharides and sodium polymethacrylate and being crosslinked by an aldehyde crosslinking agent (see JP-A-57-153658 and 57-160465) and this carrier is being used as a carrier consisting of gelatin particles instead of animal erythrocytes. The above mentioned Japanese Patent Un-examined Publications disclose that the artificial particles have properties similar to those of animal erythrocytes, which are the best conventional carriers for immunoagglutination reactions, are also chemically and physically stable, have no antigenicity, and can be easily prepared in a desired particle size in a large amount.

However, gelatin is a natural protein material and its properties change depending on the raw material so that the particles cannot always be prepared with uniform properties. Furthermore, the trend towards greater speed and automation in the field of clinical analysis makes it desirable to be able to carry out the reaction in the agglutination reaction analysis more rapidly than possible using animal erythrocytes or gelatin particles.

Therefore, the present invention aims to provide novel carrier particles which are more uniform and stable and enable the agglutination reaction to be carried out more rapidly, compared with conventional carriers. The carrier particles of the present invention are prepared from a starting material which is not a natural material but a synthetic material, i.e., polyamino acid, where the coacervation method is used as a granulation method.

Contrary to gelatin, the polyamino acid used in the present invention does not gelatinize at room temperature in the granulation process, so that it is possible to prepare coacervate particles. In this method, it is unnecessary to cool the system at the time of making the particles insolubl and also unnecessary to control the temperature of the system, since the speed of the reaction between the particles and the crosslinking agent is slow.

It is suggested in JP-A-55-94636 that synthetic polyamino acid be used as carrier particles for an antigen-antibody reaction. The publication relates to a microcapsule for use in an antigen-antibody reaction

obtained by the coacervation microcapsule technique and discloses on page 5 line 2 from the bottom, that polyamino acid resin can be used as a material capable of forming a capsule shell. How ver, this prior art substantially discloses gelatin-gum arabic capsule particles, the core of which is formed from oily material and does not specifically disclose any method for preparing capsule particles by using a particular polyamino acid under particular conditions. In fact, experiments conducted by the present inventors revealed that it was very difficult to obtain particles using a commercially available polyamino acid, for example, poly-L-glutamic acid, under coacervation.

Furthermore, JP-A-62-1728 discloses a polyamino acid spherical particle and a method for the preparation thereof and also discloses that one of its uses is as a latex for bioreaction. However, since the particles are prepared by dissolving a hydrophobic polyamino acid in an organic solvent and dispersing the thus prepared solution into an aqueous medium which is a non-solvent, the thus-prepared particles are essentially hydrophobic and have disadvantages similar to those of polystyrene latex. In addition, as is clear from the Examples set out in the specification, the spherical particles prepared by this conventional method are of a particle diameter of about 40~ 75 μm or 75 ~ 200μm. It has been confirmed that even when particles of not more than 10μm are obtained by sieving the thus obtained particles are not true spheres and do not sufficiently sensitize the antigens and antibodies.

It is therefore the object of the present invention to provide improved artificial carrier particles which overcome the disadvantages of conventional carrier particles for particle immunoassays, in particular, carrier particles which are chemically and physically stable and which are capable of producing results in a short time period in an assay such as a microplate agglutination reaction.

This object can be attained by forming particles of a complex comprising a synthetic polyamino acid having at least one carboxyl group and at least one amino group in its side chain and an anionic polymer by phase separation, i.e., coacervation, and insolubilizing the resulting particles utilizing a crosslinking agent so as to obtain artifical particles. In more detail, the artificial carrier particles of this invention can be obtained by preparing a synthetic polyamino acid having at least one carboxyl group and at least one amino group in its side chain in an appropriate ratio as described later, forming liquid drops (coacervate) of small size from the polyamino acid and a water soluble anionic polymer according to the complex coacervation method and insolubilizing the drops. In this regard, there can be obtained carrier particles having a desired particle diameter and sensitivity by appropriately controlling the ratio of the carboxyl group and the amino group present in the polyamino acid as the raw material, the mixing ratio of the polyamino acid and the anionic polymer, and the coacervation parameters, for example, the pH.

Fig. 1 represents reaction equations by which synthetic polyamino acids of the present invention having at least one carboxyl group and at least one amino group are prepared;

Fig. 2 represents another reaction equation by which a synthetic polyamino acid of the present invention having at least one carboxyl group and at least one amino group is prepared;

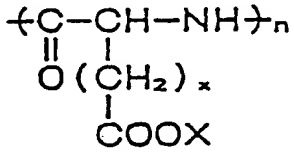
Fig. 3 also represents other reaction equations by which a synthetic polyamino acid of the present invention having at least one carboxyl group and at least one amino group is prepared;

Fig. 4 is a histogram showing the particle diameter distribution (volume distribution) of the carrier particles of Fig. 1; and

Fig. 5 is a photograph showing the structure of the carrier particles of the present invention (Sample 2).

Although the polyamino acid having at least one carboxyl group and at least one amino group in its side chain usable in this invention can be prepared by any method known in the art, one of the following three methods is generally preferable. In this connection, polyamino acids used as raw material and the method for the preparation thereof are well known and therefore, any of various kinds of the polymers and methods for the preparation thereof can be selected in accordance with the purpose.

(1) Introduction of an amino group into an acidic polyamino acid having the following formula:



X=1 aspartic acid

X=2 glutamic acid

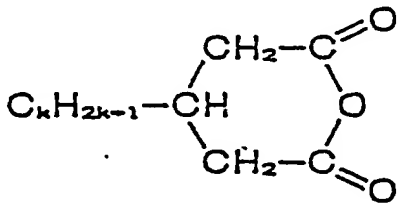
As shown by th reaction equations of Fig. 1, the amino group is introduced into the polyamino acid via a COOX group thereof by the use of a polyamine or amino alcohol having a valency of not less than two. The marks ① to ⑥ have the following meanings:

① X generally represents a protective group and usually represents a methyl, ethyl or benzyl group.

② Although a divalent aliphatic amine is shown (m = 2,3,4,6———), for convenience, an aryl amin such as phenylene diamine and polyamines having a valency of not less than three, can be used insofar as the introduction of the amino group can be accomplished for the purpose of the present invention.

③ Hydrolysis is carried out to eliminate the protective group. In this case, an alkali such as NaOH and KOH is ordinarily used.

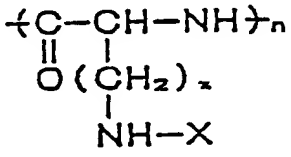
④ P is zero or 1. Alternatively, the following compound or the like can be used.



⑤ Although an aliphatic alcohol (m = 2,3,4,6,———) is shown, an aryl alcohol or the like can be used.

⑥ P-toluene sulphonic acid, HCl or the like can be used as an acid catalyst.

(2) Introduction of a COOH group into a basic polyamino acid having the following formula:



X=2 α , γ -diaminobutyric acid

X=3 ornithine

X=4 lysine

As shown by the reaction equation of Fig.2, a COOH group is introduced into the polyamino acid via the NH_2 group thereof by use of an acid anhydride. The marks ① to ② have the following meanings:

① The compound after eliminating the protective group is shown. As a rule, the NH_2 group of a basic polyamino acid is protected e.g. by carbobenzoxy (Cbz.), P-chlorocarboxybenzoxy (Clz) during the synthesis. The elimination of the protective group can be carried out by any conventional method.

② is the same as ④ of (1),mentioned above.

(3) Copolymerization of an acidic amino acid and a basic amino acid:

The copolymerization can be carried out according to the reaction equation as shown in Fig.3. As one example, Fig.3 shows the copolymerization of glutamic acid and lysine, and a similar method can be applied to any case of use of other amino acids.

A copolymer of an acidic polyamino acid and a neutral polyamino acid, a copolymer of a basic polyamino acid and a neutral polyamino acid, and a copolymer of an acidic polyamino acid, a basic polyamino acid and a neutral polyamino acid can also be used to prepare a polyamino acid having at least one carboxyl group and at least one amino group in its side chain for the present invention, through such procedures as set out in-(1), (2) and (3) above, respectively.

The various process parameters for the methods (1) to (3) are well known in the art and are described in various publications. For example, where the introduction of an amino group in the method (1) is carried out by use of ethylene diamine, hexam thylene diamine or the like as an amination agent, the diamine is added to a polymer (a commercially available polymer is usable) in an appropriate ratio and these materials are reacted in water, preferably in distilled water, for 20 minutes to 2 hours under progressively increasing

temperature, for exampl ,at the reflux temperature, and the hydrolysis is carried out in the presence of an alkali such as NaOH, preferably under progressively increasing temperature, for 2 to 20 hours, preferably 5 to 15 hours. Since th polymer unit ratio of a to b (cf. Fig. 1 and 2), i.e., the ratio of COOH groups to NH_2 groups contained in th polymer can be changed to an appropriate valu by adjusting th w ight ratio of the starting polymer to the amination agent (for the method as shown in (1),above), the weight ratio of the starting polymer to the partial carboxylation agent (the method as shown in (2), above), or th ratio of the respective polymers for the copolymerization (the method as shown in (3),above), the present invention has advantages in that good coacervation is obtained in the coacervation process using the polyamino acid together with the anionic polymer so as to prepare the final carrier particles and that the sensitivity of the final carrier particles can be controlled according to the target antigen or antibody. In this connection, the ratio between COOH groups and NH_2 groups contained in the polymer is ordinarily 10:90 to 90:10, preferably 20:80 to 60:40. While an amino acid has various polymerization degrees (n), n is ordinarily 50 to 1500, preferably 200 to 700.

As the anionic polymer which is another starting material for preparing the carrier particles, there can be used one usable in the conventional coacervation method, for example, water soluble polysaccharides such as gum arabic, carboxymethyl cellulose, sodium alginate, agar and carragenane. In addition, as the anionic polymer there can be also used , for example, polyglutamic acids such as sodium polyglutamate, polyacrylates such as partially hydrolyzed polyacrylate and polyacrylamide. Preferred is gum arabic. Any of these commercially available can be used as the anionic polymer.

In order to obtain the desired carrier particles from the synthetic polyamino acid having at least one carboxyl group and at least one amino group in its side chain and the anionic polymer, this invention is based on the complex coacervation. Thus, the polyamino acid and the anionic polymer are used as polycation and polyanion, respectively, and thereby a uniform aqueous solution is prepared within the range of concentrations at which coacervation occurs. The pH of the resulting solution is adjusted to not higher than the isoelectric point of the polyamino acid under stirring so as to obtain a coacervate. The carrier particles can be prepared by insolubilizing the resulting coacervate. In more detail, a mixture of the polyamino acid and the anionic polymer having a weight ratio of about 5:1 to about 1:5, preferably about 2:1 to about 1:2, is dissolved in water, preferably distilled water, in a concentration of about 0.05 to 5 weight %, preferably about 0.1 to 3.0 weight % to obtain a solution with a pH of about 9 to 12. If necessary, insoluble materials can be removed therefrom by filtration or centrifugation. The pH of the solution is then adjusted to 3.0 ~ 9.0 by addition of an acid such as hydrochloric, sulfuric or acetic acid at room temperature or under progressively higher temperature (20 ~ 40 °C), under stirring, to obtain coacervates, and the resulting coacervates are insolubilized by crosslinking them with a crossliking agent. The pH range for forming the coacervates is determined in accordance with the ratio of COOH groups to NH_2 groups of the amino acid used, the mixing ratio of the polyamino acid and the anionic polymer and the desired particle size. Therefore, according to this invention, properties and particle size of the thus obtained paricles can be optionally controlled. For example, particles of large particle size can be prepared by lowering the pH while particles of small particle size can be prepared by raising th pH, and thereby, the particle size of the particles can be controlled to 2 ~ 10 μm, which is suitable for use in a microtiter plate agglutination reaction or to 0.1 ~ 1.0 μm which is suitable for use in a latex agglutination reaction. The thus-prepared particles have an electric double layer similar to that of animal erythrocytes and thus have stable dispersability and good agglutination properties at the time of conducting the assay.

Examples of aldehyde crosslinking agents usable for insolubilizing the particles include ordinary ones usable in the crosslinking of the coacervate, for example, glutaraldehyde, formaldehyde, glyoxal, crotonyl aldehyde, acrolein and acetoaldehyde, the preferred one being glutaraldehyde. The crosslinking agent is added in an amount of about 0.01 to 5.0 weight %, preferably 0.1 to 2.0 weight %, to the aqueous solution after formation of the coacervate.

In this invention, a part of the synthetic amino acid polymer can be replaced with an optional polycationic ingredient such as gelatin while a part of the anionic polymer can be replaced with an optional polyanionic ingredient such as sodium polyphosphate. Furthermore, animal erythrocytes or other suitable core materials can be present in the coacervate preparation to obtain capsule particles. In this case, since the surface (capsule membrane) of the particles consists of the synthetic amino acid and the anionic polymer, carrier particles can be prepared which are substantially identical with those described above.

Furthermore, in accordance with necessity, it is possible to use an ordinary polar solvent such as methanol, ethanol or acetone as a bad solvent at the time of the coacervation granulation for the purpose of accelerating the production of the coacervate (production of the particles) and to use a surfactant, in particular, an anionic surfactant or a nonionic surfactant for the purpose of improving the disparsability of the thus prepared particles. Although these polar solvents and / or surfactants are used in an amount

effective for th purpose, it is suffici nt that the former be used in an amount of 5 to 60 weight % of the solution for forming the coacervate and the latter be used in an amount of 0.005 to 0.5 weight %.

Although th thus obtained carrier particles of this invention are substantially colorless, the particles can, if desired, be colored by a conventional method with an ordinary dye, for example, a reactive dye such as reactive red 4, reactive red 120, reactive blue 4. or reactiv red 5, or a direct dye such as direct orange 31, direct red 31 or direct blue. Specifically, the thus prepared insoluble polyamino acid particles are immersed for one night in a solution of appropriate concentration for dying, for example, in a solution containing the dye in an amount of 0.01 to 3.0%. Alternatively, the dye can be added to the solution for forming the particles.

Sensitization of an antigen or an antibody to the particles of this invention can be easily carried out by the conventional method for sensitizing it to animal erythrocytes. For example, the particles are conventionally subjected to tannic acid treatment, after which the desired antigen or antibody is adsorbed thereon. The antigen or antibody for use in the sensitization may be an optional one from a natural source or from a product of gene recombination, cell fusion or chemical synthesis, which corresponds to the one to be analyzed.

As shown by the following examples, the sensitized carrier of this invention has properties similar to those of conventional animal erythrocytes and gelatin carriers and also has novel characteristics in that the sedimentation rate after the agglutination is high, so that it is possible to shorten the time period for the assay. Although the reason for their high sedimentation rate is not clear, it is estimated that it is related to the specific gravity of the carrier particles of this invention because the specific gravity of the carrier particles of this invention is larger than that of animal erythrocytes or gelatin.

The present invention and characteristics thereof are explained with reference to the following non-limitative examples. Referential Example 1

Preparation of polyglutamic acid to which an amino group is introduced.

According to the conventional method, powders of poly-γ-methyl-L-glutamate were prepared by reprecipitating from methanol Ajicoat A-2000 (which is a 10 weight % solution of poly- γ-methyl-L-glutamate, the polymerization degree being about 580, and is commercially available from Ajinomoto Co. Ltd.) and drying the resulting material. 70 g of the resulting powders were charged in a 2ℓ flask provided with a condenser, thermometer and stirrer, which contained therein ethylene diamine and distilled water in a ratio listed in Table 1, and were heated at the reflux temperature for about 1 hour. 350 ml of 2 weight % NaOH aqueous solution was taken added thereto and the reaction was continued until the reaction solution became substantially transparent, after which 14 g of solid NaOH was added thereto. The resultant was directly subjected to suction filtration using a cotton cloth to remove any insoluble material therefrom and further concentrated by a vaccum evaporator to remove water and residual ethylenediamine, whereby about 200 ml of viscous material was obtained. The viscous material was dissolved in about 0.7 to 1 ℓ of ethanol and the resulting solution was added to 2 to 3 ℓ of ether to prepare flake materials. The materials were filtrated by suction , washed with an appropriate amount of a mixture of ethanol and ether in a ratio of 1:3 and an appropriate amount of ether in this order, and dried under vacuum to obtain each sample in an amount of about 50 to 70 g.

Table 1

Sample No.	Ethylenediamine	Water
1	500 ml	500 ml
2	600 ml	400 ml
3	700 ml	300 ml
4	800 ml	200 ml
5	300 ml	700 ml
6	400 ml	600 ml

Another sample, Sample 7, was also prepared without addition of ethylenediamine, i.e., the hydrolysis was only conducted until the reaction solution became transparent.

Regarding these Samples 1 to 7, the viscosity of 1M acetic acid aqueous solution containing the sample in an amount of 0.01g/1 ml was measured and the ratio of COOH groups to NH_2 groups (a:b) was also determined by NMR spectroanalysis. The results obtained are shown in Table 2.

Table 2

Sample No.	Viscosity (cp)	a:b
1	1,330	51:49
2	1,305	39:61
3	1,306	31:69
4	1,308	12:88
5	1,308	73:27
6	1,296	60:40
7	1,256	100:0

Referential Example 2

Preparation of poly-L-lysine into which a carboxylic group is introduced.

10g of poly (L-lysine) bromate (which is available from Seikagaku Kogyo K.K and has a polymerization degree of about 300) and 14 ml of triethylamine were added to 100 ml of dimethylformamide and stirred at room temperature for 1 hour, after which 2.7 g of succinic anhydride was added thereto. After stirring at room temperature for 6 hours and further stirring at a temperature of 60° C for 1 hour, the solution was concentrated to 20 ml by an evaporator. 100 ml of ethanol in which 3.8 g of NaOH was dissolved was added to the resulting concentrate, sufficiently mixed by shaking, after which the resultant was left to stand in a refrigerator for one night. The solid was collected by filtration, sufficiently washed with ether and dried. 7.6 g of the desired polymer was obtained (yield was 90 % as sodium salt of the polymer) and the ratio of amino groups to carboxyl groups was determined to be about 60:40 by NMR spectroanalysis. The thus obtained polymer was used as Sample 8. Referential Example 3

Preparation of poly-L-glutamic acid and poly-L-lysine copolymer

30,6 g of N^{ϵ}-carbobenzoxy-L-lysine N^{ι}-carboxy anhydride prepared by the conventional method (phosgene method) and 26.3 g of γ-benzyl-L-glutamic N-carboxy anhydride were dissolved in 600 ml of tetrahydrofuran.

0.15 ml of triethylamine as polymerization initiator was added thereto under stirring at room temperature. After stirring for two days, the solution was concentrated to 100 ml under vacuum by an evaporator and 1000 ml of water was added thereto to obtain white precipitates. The precipitates were washed with methanol and water, and dried. 43.3 g of the copolymer was obtained at a yield of 90% as 1:1 copolymer. The thus obtained copolymer was dissolved in 200 ml of a mixed solution of trifluoroacetic acid and hydrobromic acid, stirred at room temperature for 1 hour, after which 500 ml of ether was added thereto to obtain yellow brown precipitates. The precipitates were collected by filtration, sufficiently washed with ethanol and ether, and dried to obtain 27.5 g of the desired copolymer (yield was 85%, based on the starting material). In this case, it was confirmed by NMR spectrolanalysis that the residual lysine of the thus obtained copolymer was formed as hydrobromate and that the ratio of lysine to glutamic acid was 50:50. The resultant copolymer was further dissolved in water and treated with NaOH to prepare the Na salt thereof. After reprecipitating from ether, the resultant copolymer (Sample 9) was used in the latter process of coacervation.

Example 1

Preparation of carrier particles

Granulation of Samples 1 to 9 prepared in Referential Exampl s 1 to 3 was carried out by the following method:

(1) In case of Samples 1 to 3, 6, 8 and 9:

1.0 g of each sample, 20 ml of 5% gum arabic aqueous solution and 180 ml of distilled water were sufficiently mixed in a beaker and 1.5 ml of 2 weight % active red 120 aqueous solution was added thereto to prepare each stock solution. The pH of the stock solution was controlled with 10 weight % acetic acid solution at room temperature under sufficient stirring, and the formation and the particle diameter of the solution drops (coacervates) were checked by an optical microscope. 2.5 ml of 25 weight % glutaraldehyde (GA) aqueous solution was then added thereto and aggitated at room temperature for about 1 hour followed by further aggitation at a temperature of about 40 °C for about 1 hour. The resulting particles were sufficiently washed with distilled water three times. Centrifugal separation (for 5 minutes at 2000 rpm) was used to recover the residue. The pH of each stock solution relative to each Sample and the pH of the solution when the coacervates having the desired particle diameter were formed are shown in Table 3.

Table 3

Sample No.	pH of the stock solution	pH of the solution when the coacervates were formed
1	10.92	5.63
2	11.02	7.56
3	11.58	8.18
6	10.97	4.57
8	11.05	7.50
9	10.95	6.01

The ratio of particles in the range of 2 to 7 μm contained in each Sample was determined by a particle size distribution analyzer available from Galai Vo. Ltd. In each case the ratio was found to be in the range of 93 to 94 % (volume distribution). A histogram and a microscopic photograph of Sample 2 are shown in Figs. 4 and 5.

The degree of the electrophoresis of each carrier particle of the Samples set out above was determined with a system 3000 Automated Electrokinetics Analyzer manufactured by PENKEM, Inc. For this, the particles were suspended in a solution containing 0.15 M PBS and having a pH of 7.2 and the suspension was charged in a 1 mm ϕ x 20mm cylindrical electrophoresis cell to measure the degree of electrophoresis under the gradient voltage of 1048 V/m at room temperature. It was found that the degree of electrophoresis of each particle was in the range of -0.87 to -1.20μm/sec/V/cm and that the degree was the same as -1.15 μm/sec/V/cm of sheep erythrocytes and -0.75 to -1.85 μm/sec/V/cm of gelatin particles determined in a manner similar to that of the method set out above, which data were reported in publications such as Rinshokensa Vol. 30 No. 13, 1709-1710 and Japanese Patent Un-examined Publication No. 57-153658.

Furthermore, the specific gravity of each particle was determined by the following method. First, sucrose solutions of 20 w/w %, 30 w/w %, 40 w/w %, 50 w/w %, 60 w/w % and 65 w/w % were prepared and 2 ml of the respective soutions were charged in a test tube in the order of their weight, with the heaviest one being charged first. 0.5 ml of 1 % carrier particle floating solution was added to the upper most layer of the sucrose solution of 20 w/w %. The test tube was then subjected to centrifugal separation at 3000 rpm for 20 minutes. After centrifugation, all carrier particles were found to be present in the sucrose solutions of 60 w/w % and 65 w/w %. The same experiment was conducted for fixed sheep erythrocytes,in which case the erythrocytes were found to be present in the sucrose solutions of 60 w/w % and 50 w/w %. This r sult shows that the polyamino acid carrier particles have a higher specific gravity than that of the fixed sheep erythrocytes which are similar to those of gelatin particles.

(2) In case of Sample 4:

Carrier particles having a particle size distribution substantially the sam as that of the particles of item (1) were prepared by the sam granulation method as that of item (1) except that the amino acid sample was used in an amount of 0.5 g.
pH of stock solution 11.36
pH of the solution for forming the desired particle diameter 9.23

(3) In case of Sample 5:

Carrier particles having the particle diameter distribution substantially the same as that of the particles of item (1) were prepared by the same granulation method as that of item (1) except that a mixture of 100 ml water and 80 ml ethanol was used instead of 180 ml water.
pH of stock solution 10.97
pH of the solution for forming the desired particle diameter 5.88

(4) In case of Sample 7:

Conditions such as pH and amount of added bad solvent were variously changed, but the desired particles could not be formed.

(5) In case of Sample 10:

Carrier particles having a particle diameter distribution substantially the same as that of the particles of item (1) were prepared by the same granulation method as that of item (1) except that an aqueous solution containing sodium polyglutamate (MW : 7200) was used instead of 5% gum arabic aqueous solution.

(6) In case of Samples 11 and 12:

Carrier particles comprising a copolymer of glutamic acid and leucine and having a particle size distribution substantially the same as that of the particles of item (1) were prepared by the same granulation method as that of item (1) except that the following raw materials were used.

Glutamic acid	98 mol %	95 mol %
Leucine	2 mol %	5 mol %
Ratio of amino acid groups to carboxy groups	65:35	56:44
pH of stock solution	11.14	11.32
pH of the solution for forming the desired particle size	6.52	7.01

Example 2

Preparation of carrier particles

In the granulation method of item (1), Sample 2 was used without addition of the dye and glutar aldehyde was added to the solution when the pH of the solution reached 7.91, followed by centrifugal separation at 2500 rpm for 10 minutes to collected the supernatant liquid. The collected supernatant was filtrated using a 1μm membrane filter.

The filtrate was subjected to centrifugal separation at 4000 rpm for 10 minutes to obtain particles. The particle size of the thus obtained particles was determined by a particle diameter distribution analyzer CIS-1 manufactured by Galai Co.Ltd. As a result, it was found that about 80% of the particles had a particle diameter in the range of 0.5 to 1.0μm (volume distribution). These particles are usable as particles in latex

agglutinaion such as the Turbidimetric immunoassay.

Example 3

With carrier particles of the present invention as prepared by the method in Example 1, as well as fixed sheep erythrocytes as reference, particle agglutination tests were done by the conventional microtiter method. For the tests, the carrier particles (and the fixed sheep erythrocytes) were subjected to a conventional sensitization treatment with tannic acid, and then sensitized (coated) with the sensitizing materials (antigens or antibodies) as shown in Table 4. The specimens for testing are human sera which are positive or negative with respect to the components corresponding to the respective sensitizing antigens or antibodies. Thus, for example, in the case of the test of HBs antigen sensitized carrier, anti-HBs antibody positive human serum was used as positive specimen and anti-HBs antibody negative human serum was used as negative specimen. The exception was the use of anti-BSA rabbit immune serum as positive specimen for the bovine serum albumin (BSA) sensitized carriers.

The tests were carried out firstly by adding 25 µl of diluent to each well of the microtiter plate. Then, 25 µl of each specimen was serially diluted (through the two-fold dilutions) in the wells, and mixed with 25 µl of a 1v/v% suspension of the sensitized carrier particles (or the sensitized fixed sheep erythrocytes as reference). The mixture was allowed to stand for two hours at room temperature. The highest dilution giving a positive reaction (agglutination reaction) was determined to be the endpoint (agglutination titer).

The conditions for the sensitizations as described above were as follows:
One milliliter of a 2.5% suspension (in PBS, pH7.2) of the carrier particles (or the fixed sheep erythrocytes) was mixed with an equal volume of tannic acid solution (in PBS) and the mixture was allowed to stand for 30 min. at 37 °C. After removal of the supernatant by centrifugation, the particles were washed with PBS, and then suspended in 1ml PBS. One milliliter containing each amount of antigen (or antibody) source was added to each preparation and then incubated at 37 °C for 30 min. After the incubation, the supernatant was removed by centrifugation. The particles were washed with PBS 3 times, and suspended in 2.5ml. PBS. A 1% concentration by volume of sensitized particles was used for testing.

The results obtained are shown in Table 4. As is clear from the data in Table 4, it is understood that the carrier particles of this invention have an endpoint equal or superior to that of fixed sheep erythrocytes. In addition, it was possible that the assay was finished from about 60 to 80 minutes by use of the carrier particle of this invention, while the fixed sheep erythrocytes required about 90 to 120 minutes to finish the assay.

Table 4

Sensitizing material	Carrier	End point	
		Positive specimen	negativ specimen
HBc antigen (from recombinant yeast)	Fixed sheep erythrocytes	2^9	$< 2^1$
	carrier particles of Sample 1	2^{18}	$< 2^1$
HBs antigen (from plasma)	Fixed sheep erythrocytes	2^7	$< 2^1$
	carrier particles of Sample 3	2^7	$< 2^1$
HBs antigen (from recombinant yeast)	Fixed sheep erythrocytes	2^7	$< 2^1$
	carrier particles of Sample 3	2^7	$< 2^1$
HBs antibody (guinea pig)	Fixed sheep erythrocytes	2^8	$< 2^1$
	carrier particles of Sample 2	2^8	$< 2^1$
HBs antibody (mouse monoclonal)	Fixed sheep erythrocytes	2^8	$< 2^1$
	carrier particles of Sample 2	2^8	$< 2^1$
HIV antigen TFR ID = 26/011>*1	Fixed sheep erythrocytes	2^8	$< 2^1$
	carrier particles of Sample 2	2^8	$< 2^1$
Bovine serum albumin	Fixed sheep erythrocytes	2^{10}	$< 2^1$
	carrier particles of Sample 8	2^{10}	$< 2^1$

*1 This antigen was solubilized with a surfactant by the conventional method.

Claims

1. An artificial carrier particle comprising an anionic polymer and a synthetic polyamino acid having at least one carboxylic group and at least one amino group in its side chain, the complex being insolubilized by an aldehyde crosslinking agent.

2. An artificial carrier particle of claim 1 wherein the molar ratio of the carboxyl group to the amino group contained in the synthetic polyamino acid is about 10:90 to about 90:10.

3. An artificial carrier particle of claim 2 wherein the molar ratio of the carboxyl group to the amino group contained in the synthetic polyamino acid is about 20:80 to about 60:40.

4. An artificial carrier particle of claim 1 wherein the synthetic polyamino acid is an acidic polyamino acid and the amino group is introduced in the acidic polyamino acid via a part of a free carboxyl group of the polyamino acid.

5. An artificial carrier particle of claim 1 wherein the synthetic polyamino acid is a basic polyamino acid and the carboxyl group is introduced in the basic polyamino acid via a part of a free amino group of the polyamino acid.

6. An artificial carrier particle of claim 1 wherein the synthetic polyamino acid is a copolymer of an acidic polyamino acid and a basic polyamino acid.

7. An artificial carrier particle of claim 1 wherein the synthetic polyamino acid has a polymerization degree of 50 to 1500.

8. An artificial carrier particle of claim 1 wherein the anionic polymer is a water soluble polysaccharide.

9. An artificial carrier particle of claim 8 wherein the watersoluble polysaccharide is gum arabic.

10. An artificial carrier particle of claim 1 wherein the weight ratio of the synthetic polyamino acid to the anionic polymer is about 5:1 to about 1:5.

11. An artificial carrier particle of claim 10 wherein the weight ratio of the synth tic polyamino acid to the anionic polymer is about 2:1 to about 1:2.

12. A process for preparing artificial carrier particles, which comprises the steps of preparing an aqueous solution containing an anionic polymer and a synthetic polyamino acid comprising at least one free carboxyl group and at least one free amino group in its side chain, adjusting the pH of the solution to 3.5 to 9.5 at room temperature or at progressively increasing temperature under stirring to form a solution of particles of a desired size, and insolubilizing the particles by an aldehyde crosslinking agent.

FIG. 1

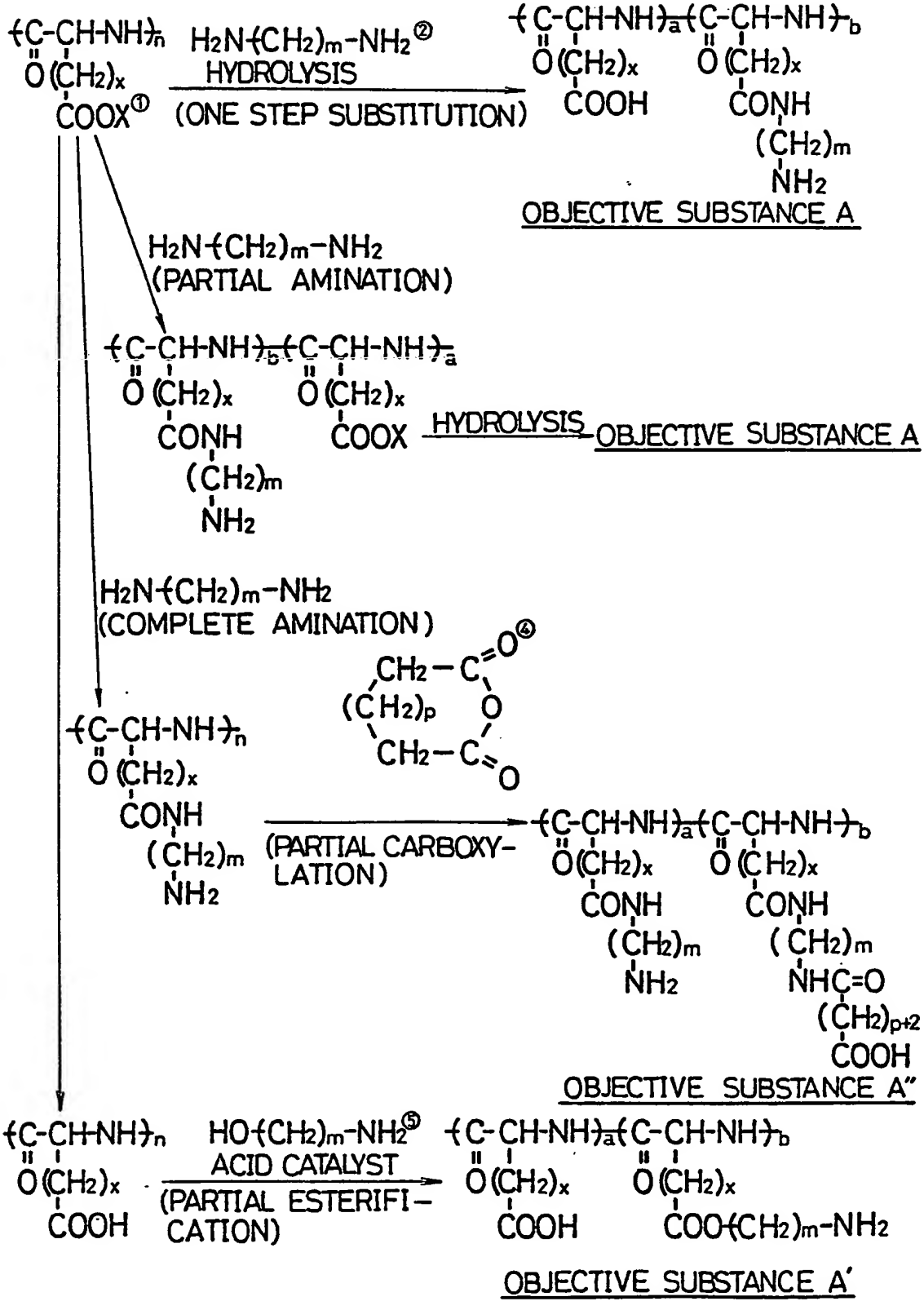
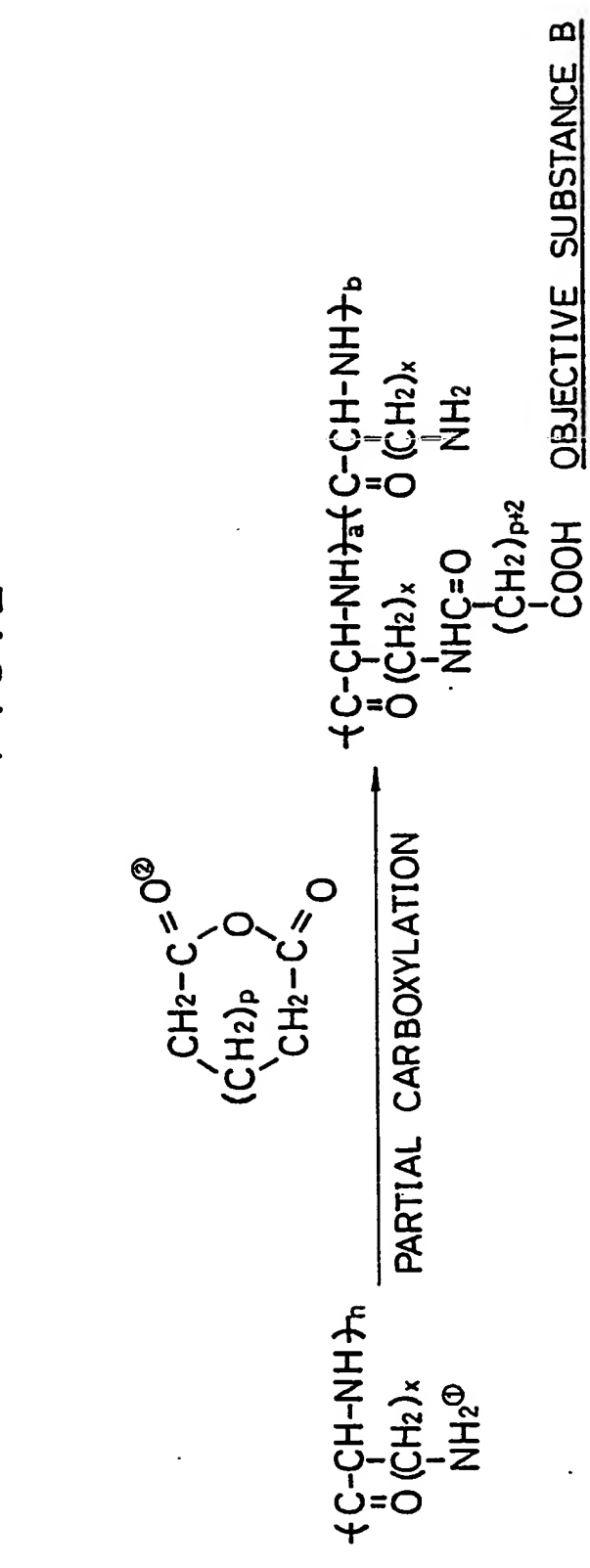

H2N-(CH2)m-NH2 ②
HYDROLYSIS
(ONE STEP SUBSTITUTION)
OBJECTIVE SUBSTANCE A
H2N-(CH2)m-NH2
(PARTIAL AMINATION)
HYDROLYSIS
OBJECTIVE SUBSTANCE A
H2N-(CH2)m-NH2
(COMPLETE AMINATION)
(PARTIAL CARBOXY-
LATION)
OBJECTIVE SUBSTANCE A"
HO-(CH2)m-NH2 ⑤
ACID CATALYST
(PARTIAL ESTERIFI-
CATION)
OBJECTIVE SUBSTANCE A'

FIG. 2



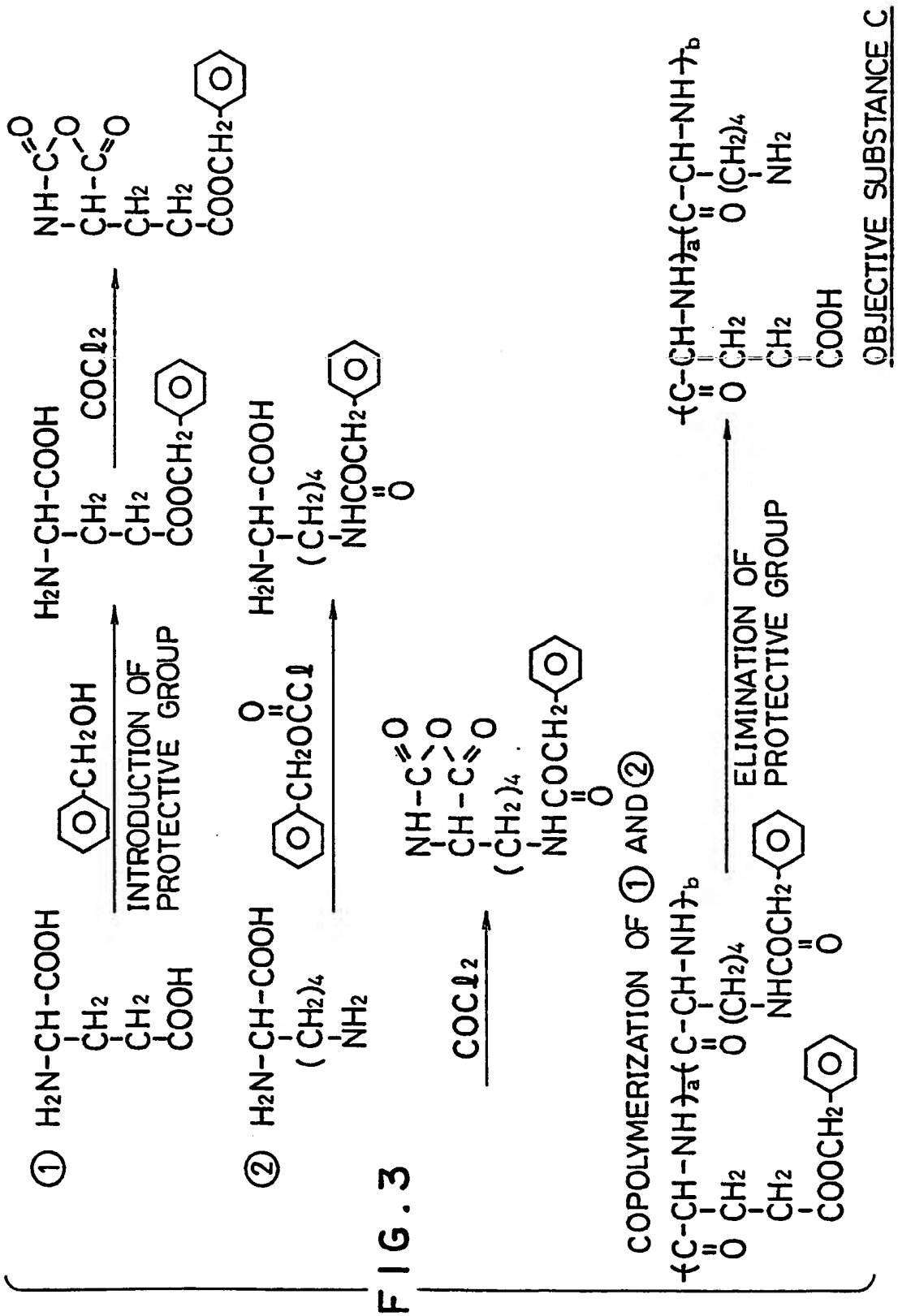

① H2N-CH-COOH
CH2
CH2
COOH
CH2OH
INTRODUCTION OF PROTECTIVE GROUP
② H2N-CH-COOH
(CH2)4
NH2
O
CH2OCCl
COCl2
NH-C=O
CH-C=O
(CH2)4
NHCOCH2
O
COPOLYMERIZATION OF ① AND ②
ELIMINATION OF PROTECTIVE GROUP
OBJECTIVE SUBSTANCE C

FIG. 3

FIG. 4

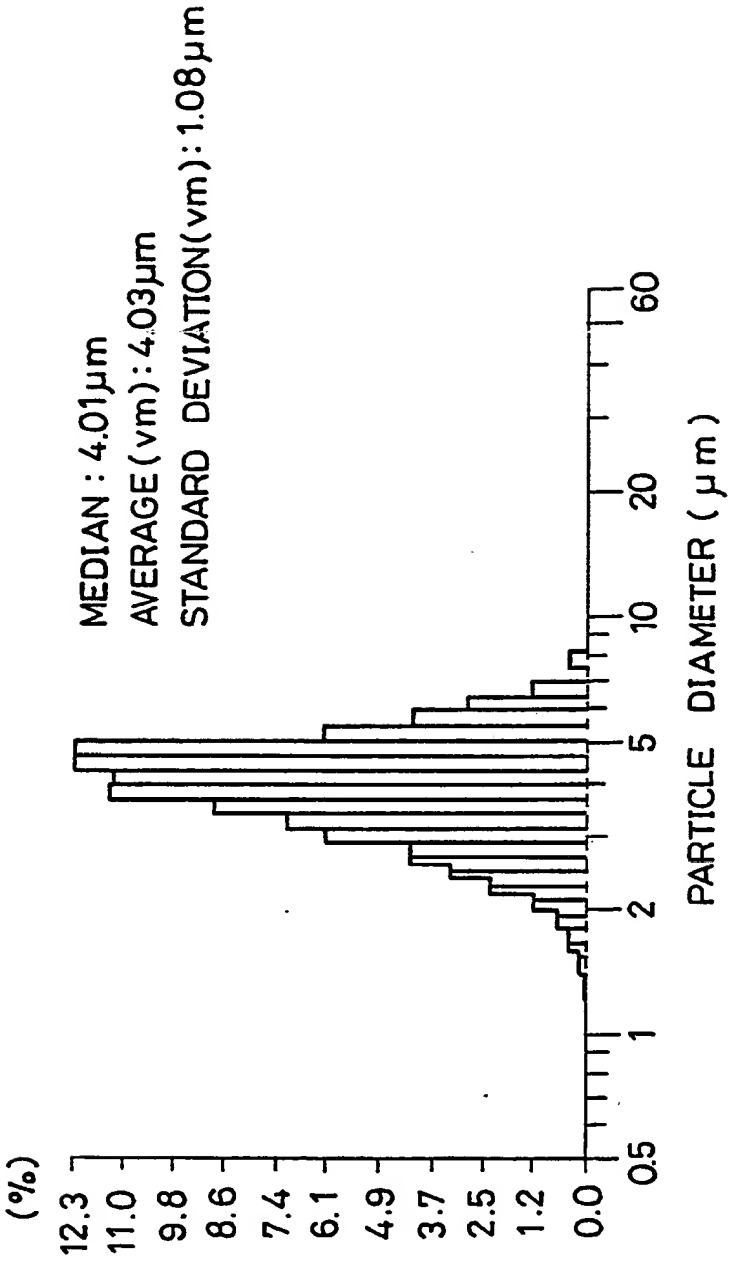
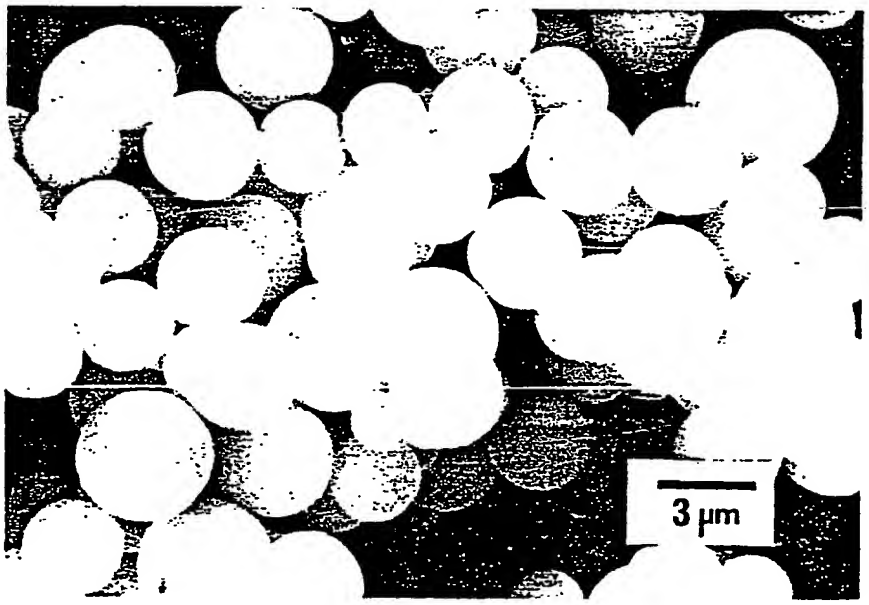

MEDIAN : 4.01μm
AVERAGE (vm) : 4.03μm
STANDARD DEVIATION (vm) : 1.08μm
(%)
12.3
11.0
9.8
8.6
7.4
6.1
4.9
3.7
2.5
1.2
0.0
0.5
1
2
5
10
20
60
PARTICLE DIAMETER (μm)

FIG.5



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(54) Whole blood immunoassay

(57) A whole blood immunoassay includes the steps of mixing a whole blood sample with sensitized insoluble carrier particles to cause an immune agglutination; diluting the resulting agglutination mixture with an aqueous solution containing an erythrocyte lysing agent to lyse erythrocytes, thereby preparing an assay sample; and determining a degree of agglutination of the assay sample.

EP 1 176 424 A2

Description

BACKGROUND OF THE INVENTION

1.Field of the Invention

[0001] The present invention relates to a whole blood immunoassay, more particularly a whole blood immunoassay by use of a particle agglutination.

2.Description of Related Art

[0002] For immunoassay on infection disease-related test items, serum has been used as a sample to be tested. However, it takes at least about 30 minutes to separate serum from whole blood, including time for blood coagulation and time for subsequent centrifugation.

[0003] Typical examples of immunoassay include a radioimmunoassay (RIA), an enzyme immunoassay (EIA), a particle agglutination immunoassay and a counting immunoassay. However, the RIA and the EIA need B(Bound form) /F(Free form) separation after antigen-antibody reaction, and therefore, require time and labor before the results of the assay are obtained.

[0004] The particle agglutination immunoassay is advantageous in that it requires only the mixing of a sample to be tested with a suspension of insoluble carrier particles (e.g., latex) sensitized with an antibody or an antigen. It does not require the B/F separation and can be performed by simple operation.

[0005] In recent years, however, highly accurate simple immunoassay techniques are demanded. Particularly it has become necessary to judge rapidly whether or not a patient is infected with virus hepatitis, HIV or the like, for example, in the case of emergency operation. Accordingly, it is demanded that assay time from collection of blood up to obtainment of assay results be shortened.

[0006] Taking the shortening of the assay time into consideration, it is more desirable to use whole blood collected from a patient than to use serum, as a sample for immunoassay. However, when whole blood is used, the presence of blood cells interferes with the detection of a degree of agglutination of particles.

[0007] In view of this, for example, Japanese Unexamined Patent Publication No. HEI 10(1998)-48214 discloses a whole blood assay using a conventional latex agglutination method. According to this disclosure, a whole blood sample is hemolyzed using with a surfactant the resulting sample is tested by a latex turbidimetric immunoassay.

[0008] However, this assay has a problem in that the surfactant which needs to be used in a sufficient concentration for hemolysis affects antigen-antibody reaction and a sufficient response cannot be obtained.

SUMMARY OF THE INVENTION

[0009] An object of the present invention is to provide a whole blood immunoassay by which the interference of blood cells is avoided without any influence on antigen-antibody reaction.

[0010] The present invention provides a whole blood immunoassay comprising the steps of mixing a whole blood sample with sensitized insoluble carrier particles to cause an immune agglutination; diluting the resulting agglutination mixture with an aqueous solution containing an erythrocyte lysing agent to lyse erythrocytes, thereby preparing an assay sample; and determining a degree of agglutination of the assay sample.

[0011] These and other objects of the present application will become more readily apparent from a further detailed description given hereinafter. However, it should be understood that the following detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0012] In the whole blood immunoassay of the present invention, the whole blood sample means blood collected from a human being or other animals but not subjected to serum or plasma separation. However, before the immunoassay of the present invention is carried out, the whole blood sample may be anticoagulated with an anticoagulant and/or diluted with a reaction buffer.

[0013] As the anticoagulants used for anticoagulating the sample, usable are those usually used for blood tests such as EDTA salt, citrates and the like. Th reaction buffer is not particularly limited, and usabl are a phosphate buffer, a Tris-HCl buffer and the like, for example. The pH of the reaction buffer may suitably be about pH 6 to 8.5. To the reaction buffer, a substance suppressing a non-specific reaction, a sensitizer and the like may be added as required. The mixture

of the whole blood with the reaction buffer may be for preparation for the subsequent immune agglutination. When the whole blood is diluted with the reaction buffer, the dilution ratio may suitably be about 5 to 100 (by volume) and may preferably be 10 to 50. Temperature and time at which and during which the whole blood is mixed with the reaction buffer may suitably be about 20 to 50°C and about 1 to 5 minutes.

[0014] The insoluble carrier particles may be particles immunized, i.e., sensitized with an antigen or antibody. As materials for the particles, synthetic polymers, typically polystyrene latex or the like may be mentioned, for example.

[0015] The size of the insoluble carrier particles is not particularly limited and any known insoluble carrier particles may be used. For example, the size may be about 0.1 to 20 μ m in diameter, preferably about 0.1 to 1.0μm in diameter. The particles preferably have a uniform diameter.

[0016] The insoluble carrier particles may be sensitized by a method known in the field of art, for example, by physical adsorption, chemical binding, etc. The antigen or antibody used for sensitizing the particles is not particularly limited so long as it can be detected by utilizing antigen/antibody reaction. The insoluble carrier particles are usually used in the form of a suspension in a solvent. The solvent may suitably be water, the above-mentioned buffer or the like. The mixture ratio of the insoluble carrier particles to the solvent is suitably about 0.1 to 1 w/v%.

[0017] As regards the immune agglutination, a latex suspension containing the sensitized insoluble carrier particles is added to the whole blood sample optionally diluted with the reaction buffer so that antigen/antibody reaction takes place. Here, the mixture ratio of the sample to the insoluble carrier particles (or the mixture ratio of the diluted whole blood sample to the latex suspension) may be about 1 : 5 to 1 : 20, for example. The reaction temperature is suitably 20 to 50 °C, and the reaction time is suitably 15 seconds to 20 minutes.

[0018] As the erythrocyte lysing agent contained in the aqueous solution used for diluting the resulting agglutination mixture, are suitably used agents capable of not only destroying membrane of erythrocytes but also dissolving or contracting the membrane. For example, usable are surfactants usually used in the field of counting blood cells for lysing erythrocytes. Particularly, water-soluble surfactants may be mentioned. The water-soluble surfactants may be cationic, anionic, non-ionic or ampholytic. Among these, those having a stronger hydrophobic nature in a hydrophobic part (a larger carbon number) are more preferable because they have a greater ability to lyse erythrocytes.

[0019] Examples of cationic surfactants include alkyltrimethylammonium salts and alkylpyridinium salts.

[0020] Examples of anionic surfactants include alkyl sulfate.

[0021] Examples of ampholytic surfactants include alkyl betaine acetates.

[0022] Examples of non-ionic surfactants include polyoxyethylenealkyl ethers, polyoxyethylenealkenyl ethers and polyoxyethylenealkylphenyl ethers.

[0023] The erythrocyte lysing agent is suitably used 2 to 10000 ppm in the aqueous solution for diluting the agglutination mixture.

[0024] The aqueous solution may also contain a salt such as sodium chloride and/or a buffer in addition to the erythrocyte lysing agent. In such cases, the amount of a substance contained may be adjusted as required according to the above-mentioned pH and the like.

[0025] In the present invention, after the immune agglutination, erythrocytes are lysed for avoiding their interference with determination before measurement. In an assay as disclosed by Japanese Unexamined Patent Publication No. HEI 10(1998)-48214 in which antigen/antibody reaction is performed after the lysis of erythrocytes, a large amount of a surfactant is required for lysing erythrocytes. In the presence of the surfactant in a large amount, the antigen/antibody reaction is influenced. In order to decrease the concentration of the surfactant used, the whole blood sample needs to be reduced or diluted, which in turn decreases the concentration of an antigen or antibody to be involved in the antigen/antibody reaction and results in a poor response. However, if the antigen/antibody reaction is firstly performed under the above-mentioned condition, the antigen/antibody reaction itself is not only affected by the surfactant but also proceeds necessarily and sufficiently. Furthermore, it is possible to detect particles without destroying an antigen/antibody reaction composite (agglutination mixture).

[0026] A method of determining the degree of agglutination of the assay sample after erythrocytes are lysed may be any known method without particular limitation. Usable is a known apparatus for determining the degree of agglutination. For example, in the case of the turbidimetric immunoassay, a spectrophotometer may be used. In the case of the counting immunoassay, a measuring apparatus using the principle of flow cytometry may be used and a commercially available flow cytometer may be used.

[0027] A PAMIA series produced by Sysmex Corporation provides apparatuses for counting immunoassay. This series is suitable because a single apparatus can perform a set of operations from mixing a sample with a buffer to calculating the degree of agglutination automatically.

[0028] The determination of the degree of agglutination using a flow cytometer can be done as follows:

[0029] Agglutinated particles and unagglutinated particles contained in the prepared assay sample are extruded little by little into a laminar flow of a sheath liquid formed in a flow cell. The particles pass through the center of the flow cell one by one in line.

[0030] The particles passing through the flow cell are irradiated with laser light. After passing through the flow cell,

the laser light is stopped by a beam stopper. Only forward scattered light is received by a photo diode. As the laser light, light having a wavelength of 310 to 1285 nm may be used, for example, 488 nm, 680 nm, 780nm, 860nm, 980nm and the like. Besides the forward scattered light, side scattered light or both the side scattered light and the forward scattered light'may be detected as scattered light.

[0031] When a particle crosses the laser light, a scattered light pulse is generated which has an intensity according to the volume of the particle. The pulse is received by a light-receiving unit. Usually, the scattered light pulse received is converted to an electric pulse. The electric pulse provides information about the particle size distribution of the particles. That is, the electric pulse has an intensity according to the volume of the particle entering within the laser light, which particle may be a single particle unagglutinated, two particles agglutinated, three or more particles agglutinated, a blood cell itself, or the like.

[0032] The electric pulses are distinguished according to their intensity, and unagglutinated particles and agglutinated particles are counted. For counting these particles, a threshold value is set for distinguishing unagglutinated particles and agglutinated particles on the basis of the intensity of the scattered light. The unagglutinated particles and the agglutinated particles give scattered light of different intensities owing to their different sizes, and can be distinguished from each other. Therefore, the threshold value is set between the unagglutinated particles and the agglutinated particles for distinguishing the unagglutinated particles from the agglutinated particles according to the intensity of scattered light.

[0033] Here, the threshold value may be set in situ, at the same time as the scattered light of the assay sample is being measured, on the basis of the measured scattered light data; may be set, after the data is obtained, on the basis of the obtained data; or may be set beforehand as an estimated threshold value from known information, accumulated past data or the like. Particularly, considering measurement errors and reproducibility, the threshold value is preferably set in situ, at the same time as the scattered light of the assay sample is being measured, on the basis of the measured scattered light data.

[0034] The unagglutinated particles and agglutinated particles can be distinguished from each other and counted with reference to the threshold value, and the degree of agglutination can be calculated.

[0035] The degree of agglutination may be calculated from the number P of the agglutinated particles obtained above and the number M of the unagglutinated particles obtained above out of all counted particles, as the ratio of the agglutinated particles, which have been involved in the antigen/antibody reaction, i.e., $P / (M + P)$, $(M + P = T)$.

[0036] If particles not to be counted such as chylomicrons are present in the sample, the particle size distribution of these particles also appear in that of the object insoluble carrier particles. In this case, the particle size distribution of the particles not to be counted can be estimated by interpolation using spline function and subtracted from the particle size distribution including both the object particles and the particles not to be counted. Thereby an approximate correction data only of the object particles can be obtained and utilized for obtaining accurate counts of the agglutinated particles and the unagglutinated particles (see Japanese Patent No. 2912413).

[0037] Also, in the present invention, the degree of agglutination is calculated, and then the concentration of the antigen or antibody may be obtained from the calculated degree of agglutination.

[0038] The concentration of the antigen or antibody may be obtained by use of a calibration curve which is produced beforehand by obtaining the relationship of the degree of agglutination of the antigen or antibody to a known concentration of the antigen or antibody (preferably, a plurality of degrees of agglutination are determined with changing the concentration).

[0039] In the case where a spectrophotometer is used for the determination, a whole blood sample, a buffer and a latex reagent are mixed, and immediately after that, the resulting mixture is diluted with the aqueous solution containing the erythrocyte lysing agent for hemolysis. The hemolyzed sample is put in a measuring cell and irradiated with light to measure absorbance. The wavelength of the light is suitably 600 to 2000 nm. The absorbance at this time is regarded as absorbance at time 0 (i.e., the antigen/antibody reaction has not been taken place yet).

[0040] Subsequently, the whole blood sample, the buffer and the latex reagent are mixed and reacted for a given time. The resulting mixture is diluted with the aqueous solution containing the erythrocyte lysing agent for hemolysis. The hemolyzed sample is measured in the same manner as described above. The degree of agglutination can be obtained from a difference between the obtained absorbance and the absorbance at time 0.

Example

[0041] In this Example, RANREAM HBsAg (produced by Sysmex Corporation) was used for preparing a sample which was subjected to the latex agglutination and then hemolyzed. PAMIA-30 (produced by Sysmex Corporation) was used for determination.

[0042] RANREAM (registered) HBsAg is a reagent kit for detecting an HBs antigen and includes a latex reagent, a buffer, a sample diluent and a calibrator, among which th latex reagent and the buffer were used in this example. The latex reagent is a 0.5 % (w/v) suspension of 0.8μm polystyrene latex sensitized with an anti-HBs antibody.

[0043] Whole blood, 10 μL, was mixed with 80 μL of the buffer (pH6) and incubated at 45°C for a minute. The latex reagent sensitized with the anti-HBs antibody, 10μL, was added thereto to start reaction at 45°C.

[0044] About 20 seconds after the reaction was started, 19 μ L of the reaction mixture were mixed with 950μL of a sheath liquid (200 ppm dodecyl sodium sulfate, 0.3 g/L aqueous solution of sodium chloride) into a 51-fold dilution to lyse erythrocytes and to prepare an assay sample.

[0045] The assay sample was introduced to an optical detector of PAMIA-30 to determine the degree of agglutination P/T (%) (T1).

[0046] About 15 minutes after the reaction was started, the degree of agglutination P/T (%) (T2) was determined after erythrocytes were lysed, in the same manner as the degree of agglutination P/T (%) (T1). T1 was the degree of agglutination in the early stage of the reaction and was used for judging whether or not the sample was within a measurement range. Usually, T2 is used as the degree of agglutination (agglutination ratio) of the sample.

[0047] On the other hand, for a comparison purpose, as a prior-art example, the whole blood sample was first hemolyzed using a buffer including 10000 ppm of dodecyl sodium sulfate necessary for lysing erythrocytes and then subjected to the latex agglutination.

[0048] About 20 seconds after the reaction was started, 19 μ L of the reaction mixture were mixed with 950 μL of a sheath liquid (0.3 g/L aqueous solution of sodium chloride) into a 51-fold dilution to prepare an assay sample.

[0049] Further, for reference, the agglutination ratio (P/T) of a serum sample was determined without dodecyl sodium sulfate contained in the buffer and in the sheath liquid.

[0050] The results are shown below.

Table 1

	Serum Sample	Present Invention	Prior Art
P/T (%)	46.03	45.38	6.00

[0051] As shown above, it has been confirmed that the antigen/antibody reaction was inhibited by interference of the surfactant in the prior-art example while the reaction was not inhibited and an accurate determination was realized in the present invention.

[0052] According to the present invention, by diluting the sample with the aqueous solution containing the surfactant to lyse erythrocytes immediately before measurement, the antigen/antibody reaction can be carried out without interference of the surfactant, and a highly sensitive measurement can be performed.

Claims

1. A whole blood immunoassay comprising the steps of:

   mixing a whole blood sample with sensitized insoluble carrier particles to cause an immune agglutination;
   diluting the resulting agglutination mixture with an aqueous solution containing an erythrocyte lysing agent to lyse erythrocytes, thereby preparing an assay sample; and
   determining a degree of agglutination of the assay sample.

2. A whole blood immunoassay according to Claim 1, wherein the erythrocyte lysing agent is a surfactant.

3. A whole blood immunoassay according to Claim 1 or 2, wherein the surfactant is dodecyl sodium sulfate.

4. A whole blood immunoassay according to any one of Claims 1 to 3 which is conducted by use of an apparatus for a counting immunoassay utilizing a principle of flow cytometry.

5. A whole blood immunoassay according to Claim 4, further comprising the steps of:

   introducing the assay sample included unagglutinated particles and agglutinated particles to a flow cell, irradiating particles passing through the flow cell with laser light, and detecting scattered light generated thereby;
   setting a threshold value for distinguishing unagglutinated particles from agglutinated particles with regard to intensity of the scattered light; and
   distinguishing and counting the unagglutinated particles and the agglutinated particles in reference to the threshold value; and
   calculating the degree of agglutination from the number of unagglutinated particles and the number of agglu-

tinated particles.

6. A whole blood immunoassay according to Claim 5 or 6, wherein the degree of agglutination is calculated by the number of agglutinated particles P / (the number of agglutinated particles P + the number of unagglutinated particles M).

7. A whole blood immunoassay according to any one of Claims 5 to 6, wherein the scattered light is forward scattered light.

8. A whole blood immunoassay according to any one of Claims 1 to 7, wherein the size of the insoluble carrier particles is 0.1μm to 20μm.

9. A whole blood immunoassay according to any one of Claims 1 to 8, wherein a mixture ratio of the whole blood sample to the insoluble carrier particles is 1 :5 to 1 :20.

10. An immunoassay according to anyone of Claims 1 to 9, wherein, in the immune agglutination of the whole blood sample with the insoluble carrier particles, the reaction temperature is from 20 to 50 °C and the reaction time is from 15 seconds to 20 minutes.

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(54) Title: SYSTEM FOR ISOLATING AND IDENTIFYING LEUKOCYTES

(57) Abstract

A method and reagent system for the rapid isolation, identification, and/or analysis of leukocytes from a whole blood sample. The reagent system includes a lytic reagent solution comprising an aqueous solution of a water soluble compound which at least partially dissociates in aqueous solution to release a proton and a counterion and which, upon contact with a blood sample, acidifies the sample to a pH in the range of from about 2.6 to 4.0. The reagent system also includes a quenching reagent solution comprising an aqueous alkaline salt solution which effectively and rapidly arrests the chemical action of the lytic reagent solution on the sample and restores the native physiological environment of the sample. The method comprises using the reagent system to selectively lyse the red blood cells in a blood sample and modify the leukocytes to enable their differentiation into five sub-populations.

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SYSTEM FOR ISOLATING AND IDENTIFYING LEUKOCYTES

BACKGROUND OF THE INVENTION

Field of the Invention This invention is directed to compositions of matter and methods employing one or more of these compositions. More specifically, this invention concerns a novel lytic reagent system for rapidly effecting hemolysis of the erythrocyte fraction of whole blood and thereby permitting the isolation of the leukocyte fraction in its native or near native state. The leukocyte fraction may then be subjected to further study or analysis in a variety of environments. The lytic reagent of this invention consists essentially of a water soluble compound which at least partially dissociates in aqueous media, thereby releasing a proton and a counterion. When the appropriate concentration of such compound is added to a whole blood sample, the extent of dissociation is effective to acidify the sample (pH in the range of from 2.6 to about 4.0), while maintaining the osmolality of the sample below about 100 mOs. One of the preferred uses of this lytic reagent system is the pretreatment of the whole blood sample to effect rapid and essentially complete hemolysis of the erythrocyte fraction. Such pretreatment also results in subtle modification to the leukocyte fraction, thus, facilitating its further differentiation into at least five (5) distinct sub-populations. This reagent system is, thus, suitable for preparation of a whole blood sample for analysis on a focused flow analysis system, such as the VCS whole blood analyzer and the Epics Model C and PROFILE flow cytometer (all of which are available from Coulter Electronics, Inc., Hialeah, Florida.

Description of the Prior Art - The separation of complex biological fluids (i.e. whole blood) into its various constituents preliminary to study and analysis of its component parts, is generally desirable and often an essential requirement of many established analytical protocols and/or instrumentation utilized in such studies and analysis. Where such study or analysis of the fluid fraction of the sample is of primary interest, the cellular fraction is separated from the sample without regard for maintaining the cell viability or membrane integrity. Conversely, where the cellular fraction is itself of primary interest to the researcher or clinician, the partitioning of the whole blood sample into its various cellular components requires that the sample treatment/processing techniques be adjusted accordingly. The traditional methods for separation of whole blood samples into its various cellular components is by centrifugation. While this process is effective, it is labor intensive, relatively inefficient and requires physical manipulation of the cellular fraction of the sample.

Where such separation/partitioning of the cellular fraction of the sample is attempted with chemical agents, the results have been less than totally satisfactory for a variety of reasons. More specifically, the vitality and viability of a cell population in vivo or in vitro is dependent upon maintaining a precise physiological environment that is consistent with the preservation of physical cell structure and chemical balance within the cell. This balance is controlled by the permeability and transport characteristics of the cell membrane. Alteration in the physiological environment will in turn evoke a response or change in the cell membrane. The membrane response is "defensive" in nature; that is, the physiological response of the membrane is calculated to maintain the chemical balance within the cell and, thus, its continued and uninterrupted vitality.

It is fully appreciated that such alteration in the ideal physiological environment of the cell can be tolerated only within limits; and, that when such limits are exceeded, permanent injury to the cell can occur. As is further appreciated in the art, such changes in environment (i.e. toxicity of the diluting medium - even with distilled water) can effect hemolysis of the cells.

The degree of tolerance of various cell populations in whole blood to changes in their physiological environment has been extensively studied and documented. The effects of alteration in various aspects of the physiological environment of the cellular fraction of whole blood are both subtle and dramatic and can be effected through dietary metabolites and/or foreign substances. These studies include monitoring the reaction of cellular preparations to different drugs, Da Costa, A.J. et al, Transfusion, (1973), 13, 305; to dietary imbalance, Kobayashi, T. et al, Journal of Biochemistry (1983) 93, 675; and to changes in pH, Rother, U. et al, Z. Immunologie Forschungsgemeinschaft (1978) 155, 118, and Schettini, F. et al, Acta Paediat. Scand. (1971), 60, 17.

In each of the articles noted above, a drug, food metabolite or change in pH resulted in significant alteration in the physiological environment of the blood to the degree where hemolysis of the red blood cells was effected.

More specifically, the above referenced Rother article reports serum activation by acidification (pH 6.4) with hydrochloric acid which lysed unsensitized erythrocytes in the presence of EDTA. The article compares the effect of such acidification with the "deviated lysis" activity observed following serum activation with insulin. An independent and unrelated study by Schettini and his

co-workers concluded that red blood cells from infants and young children were more sensitive to acid hemolysis than red blood cells from older individuals.

As of the present, no chemical treatment is available to rapidly and effectively partition a whole blood sample into viable cellular fractions. Where one or more chemical treatments of the whole blood sample is used (as in the preparation or pretreatment of whole blood for the performance of white blood cell differentials), the focus of such treatment has been to alter the sample to permit the differentiation of its cellular components from one another based upon isolation/analysis of (i) the cellular debris (i.e. nuclei) remaining subsequent to such treatment; (ii) the fixation of the cells by such chemical treatments; or (iii) the relatively severe modification of such cells which eventually results in their ultimate disintegration. Such relatively harsh and disruptive chemical treatments of whole blood samples has, however, been successfully applied where combined with relatively sophisticated instrumentation. More specifically, the ability to alter the physiological environment of a cellular population in vitro has been used to advantage in the measurement of certain cellular parameters and to quantitate the individual populations. The distinctive reaction of each individual cell population to a change in its physiological environment has particular advantages in the identification of the individual leukocyte sub-populations of cells of whole blood. The leukocyte population of blood has been classified previously into two major fractions: the lymphoid and the myeloid fraction. The lymphoid fraction consists of lymphocytes (B and T cells). The myeloid fraction consists of monocytes and granulocytes (neutrophils, basophils and eosinophils). One accepted technique for modification of the physiological environment of the cellular population of whole blood has been through the addition of certain so-called "lytic reagents" to a blood sample. The

development of certain lytic reagents and lytic reagent systems has provided the clinician with the ability to effectively isolate the white cell population (hereinafter "leukocytes") from the red cell population of whole blood. The relative concentration of leukocytes within the blood sample and the gross morphological appearance of certain classes of these cells can be clinically significant.

This ability to further differentiate the leukocyte population, thus, provides an invaluable diagnostic tool in the study and treatment of various diseases. As is further appreciated, the larger the number of sub-populations of leukocytes which are identifiable, the more accurate and reliable the identification of any one such sub-population.

A number of references have appeared in the recent patent literature which disclose various reagent systems and techniques for enhancing the ability of automated instrumentation to conduct white blood cell differentials. The following references are representative of the pertinent patent literature in this field: U.S. Patents 3,874,852; 4,286,963; 4,346,018; 4,485,175; 4,520,274; 4,529,704; and, U.S. Applications Serial No. 615,961 (corresponding International Application PCT/US85/00954, published December 19, 1985); and, Serial No. 615,966 (corresponding International Application PCT/US85/00868, published December 19, 1985), all of which are hereby incorporated by reference in their entirety.

U.S. Patent 3,874,852 (to Hamill) describes a reagent system and method useful in the performance of leukocyte and hemoglobin determinations of whole blood. This reagent system comprises an essentially ferrocyanide free aqueous solution of quaternary

ammonium salt and cyanide ions. This reagent system is effective to stromatolyze both red blood cells and platelet cells in whole blood and in the conversion of the free hemoglobin to a chromagen. This system is reported efficacious for leukocyte and hemoglobin determinations with diagnostic accuracy. The leukocyte population profile available with this system is, however, limited to total white cell count, without further differentiation of this cellular fraction into its discrete sub-populations.

U.S. Patent 4,286,963 (to Ledis, et al) describes a lytic diluent and method for achieving rapid lysis of red blood cells in whole blood. This diluent enhances the ability of automated instrumentation to perform differential determinations of lymphoid and myeloid sub-populations of leukocytes and the quantitative determination of hemoglobin. The lytic diluent described by Ledis is composed of a mixture of at least one quaternary ammonium salt and an aryl substituted short chain alkanol in buffered aqueous medium (pH 3.5 to 5.0). The lytic diluent of this Ledis patent is, however, limited in its ability to effect differentiation of the leukocyte population into the two (2) principle sub-populations; namely, the lymphoid and myeloid fractions.

U.S. Patent 4,346,018 (to Carter, et al) describes a multipurpose blood diluent and a method for utilizing this diluent in combination with a weak lysing reagent system for the performance of hemoglobin determination and the differentiation of lymphocytes into the lymphoid and myeloid sub-populations. This diluent comprises, among other constituents, N-(2-acetamido)iminodiacetic acid (ADA) as a blood stabilizing agent. The lysing agent comprises an aqueous solution of at least one quaternary ammonium salt. The diluent/lytic reagent of this Carter patent is, however, limited in its ability to effect

differentiation of the leukocyte population into the two (2) principle sub-populations; namely, the lymphoid and the myeloid fractions. In addition, ADA has been found to help stabilize the size distribution, cellular shape, and most importantly, the high degree of cellular dispersion of erythrocytes and platelets to an extent not previously observed with other compounds.

U.S. Patent 4,485,175 (to Ledis, et al) describes a reagent system and method for performance of differential determinations of leukocytes into three (3) sub-populations utilizing automated cell counting equipment. This reagent system comprises a blood diluent and a lysing agent. The lysing agent (comprising an aqueous mixture of quaternary ammonium salts), when added to the diluted blood sample under mild conditions of concentration and at a relatively slow rate, causes unexpected volume modifications to the various leukocyte sub-populations. The discovery which permitted the attainment of the degree of differentiation of the leukocyte population by Ledis, et al is based upon the observation of the relative greater sensitivity of the granulocyte sub-population to lytic agents. By controlling the rate of exposure of the lymphocyte population to lytic agents, the granulocyte sub-population is better preserved. The reagent system of this Ledis et al patent is, however, limited in its ability to effect differentiation of the leukocyte population into three (3) sub-populations; namely, lymphocytes, monocytes and granulocytes.

While all of the above lytic agents and reagent systems facilitate the differentiation of the leukocyte fraction of a blood sample (to a greater or lesser degree), each suffers from a common deficiency; namely, the inability to effect such differentiation without adversely altering the chemical balance of the cells which are subjected to such treatment. Where such alteration in the

chemical balance is induced, the effect on the cellular population can range from relatively minor changes (i.e. swelling) to lysis. Dramatic chemical changes in the physiological environment of the leukocyte population also alters the immunochemical response of the leukocyte surface markers. The treatment of leukocytes with such traditional lytic agent system is, thus, inherently incompatible with further immunochemical study of these leukocytes. This limitation has thus, up to now, prevented the use of lytic reagents, alone or in combination with other means, for further refinement in the diagnostic process of various disease states, based upon the differences in the immunochemical response of the respective surface markers of each such cell population.

OBJECTS OF THE INVENTION

Accordingly, it is the object of this invention to remedy the above as well as related deficiencies in the prior art.

More specifically, it is the principle object of this invention to provide a chemical treatment, or pretreatment, of a complex biological fluid sample, such as whole blood, which facilitates the subsequent isolation, identification and/or analysis of one or more cellular populations that are present in the fluid sample.

It is another object of this invention to provide a chemical treatment, or pretreatment, of a whole blood sample which facilitates the partitioning of the sample and thereby the subsequent isolation, identification and/or analysis of the leukocyte fraction based upon the physical, physiological and/or

immunochemical properties of such fraction in its native or near native state.

It is yet another object of this invention to provide a chemical treatment, or pretreatment of a whole blood sample which is selective for only one of the cellular constituents of sample.

It is still yet another object of this invention to provide a reagent system which can rapidly and efficiently partition a whole blood sample into an essentially intact leukocyte fraction and a lysed erythrocyte fraction.

It is an additional object of this invention to provide a lytic reagent system for use in differential determination of leukocyte sub-populations of whole blood.

It is another additional object of this invention to provide a novel lytic reagent system which includes both a lytic reagent and a companion quench for use in differential determination of leukocyte sub-populations in whole blood.

It is yet another additional object of this invention to provide a novel lytic reagent system which is effective for use in differential determination of leukocyte sub-populations in whole blood by (a) measurement of physical and/or optical properties of such sub-populations, and/or (b) observation of the immunochemical response or interaction of such sub-populations with immunoreagents (i.e. antiserum) specific for one or more surface markers on each such cell sub-populations.

It is a further object of this invention to provide a method for performance of differential determination of leukocyte sub-populations by both (a) measurement of their physical and/or optical properties, and (b) immunochemical response of such sub-populations to antiserum specific for one or more surface markers on each said sub-populations.

SUMMARY OF THE INVENTION

The above and related objects are achieved by providing a chemical reagent system which is selective in its interactions with the various cellular components of a complex biological fluid sample. This reagent system will, in the various environments contemplated for its use, provide at least one reagent component to effectively alter, on a selective basis, one or more cellular constituents of the complex biological fluid sample to the degree necessary to permit the subsequent isolation, identification and/or analysis of the cellular constituents of interest. This reagent system further contemplates the ability to modulate the chemical treatment of the cellular constituents of the sample by providing a separate reagent which is designed to quench or retard the action of the cell altering reagent on the cellular populations of the sample.

The principles and concepts of this invention have been successfully applied to the treatment of whole blood samples preliminary to white blood cell differential analysis. The basic components of the chemical reagent system of this invention include a "lytic reagent" and a companion reagent for the lytic reagent referred to as a "quench." The primary functions of the quench are to retard the activity of the lytic reagent and to

restore the ionic balance of the sample subsequent to its treatment with the lytic reagent.

The lytic reagent system and method of this invention, thus, has as its broadly stated objectives, the selective hemolysis of the erythrocyte population of a whole blood sample, while facilitating the subsequent isolation, identification and/or analysis of one or more of the leukocyte sub-populations of the same sample, based upon one or more physical, physiological and/or immunochemical characteristics which are indicative of the sub-population of interest. The lytic reagent of this invention consists essentially of a water soluble compound which at least partially dissociates in the aqueous media, thereby releasing a proton and a counterion. When the appropriate concentration of such compound is added to a whole blood sample, the extent of dissociation of such compound is effective to acidify the sample (pH in the range of from 2.6 to about 4.0), while maintaining the osmolality of the sample below about 100 mOs. There are at least three (3) classes of compounds which have been found suitable as lytic reagents for achieving the objectives of this invention. These classes include low molecular weight carboxylic acids, sulfonic acids and activated phenols.

The carboxylic acids suitable as lytic reagents of this invention can be represented by the following formula

R - COOH

wherein R is H, an aliphatic hydrocarbon radical having from 1-3 carbon atoms; a carbonyl substituted aliphatic hydrocarbon radical having from 1-3 carbon atoms; a hydroxy substituted aliphatic hydrocarbon radical having from 1-3 carbon atoms; or an aliphatic hydrocarbon

radical having from 1-3 carbon atoms and multiple carbonyl and/or hydroxy substituents.

Representative carboxylic acids within the foregoing formula include formic acid, methane-carboxylic acid, (acetic acid), 2-hydroxy-ethane-2-carboxylic acid (lactic acid); 1,2-ethane-dicarboxylic acid (succinic acid); and 2-hydroxy-1,2,3-propane-tricarboxylic acid (citric acid); and their respective mixtures.

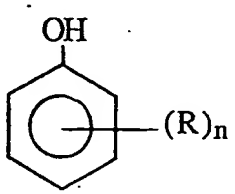
The sulfonic acids suitable as lytic reagents of this invention can be represented by the following formula

$$R\text{-}SO_3H$$

wherein R is hydroxy, an aliphatic hydrocarbon radical of 1-3 carbon atoms or aryl.

Representative sulfonic acids within the foregoing formula include sulfuric acid; methanesulfonic acid; ethanesulfonic acid; benzenesulfonic acid; p-toluenesulfonic acid; nitrobenzenesulfonic acid, and their respective mixtures.

The activated phenols suitable as lytic reagents of this invention can be represented by the following formula



wherein R is an electron withdrawing group such as halogen, cyano, nitro or any combination of such electron withdrawing substituents; and
n is 1-3.

Representative activated phenols within the foregoing formula include para-nitrophenol; meta-nitrophenol; ortho-nitrophenol; 2,4-dinitrophenol; para-chlorophenol; para-cyanophenol; and 1-chloro-2,4-dinitrophenol; and their respective mixtures.

The foregoing are representative of the classes of materials which can be used as lytic reagents consistent with the objectives of this invention. It is appreciated that the relatively weak acids will exist as both an undissociated compound and in the dissociated state within the sample. Strong acids are, of course, essentially completely dissociated in the sample. Certain undissociated acid and the counterions from the dissociated acids can apparently influence the degree of differentiation of the leukocyte fraction, depending upon their relative concentration in the sample and the physiological recognition (if any) of the acid and/or its counterion by the cellular analytes of interest. For example, phosphoric acid, even at the appropriate pH, is generally not acceptable to achieve differentiation of the leukocyte into five (5) sub-populations. It is hypothesized that counterion compatibility (phosphate ion) is unacceptable and, thus, differentiation of the leukocyte population is decidedly more difficult.

In this preferred embodiment of the concepts of this invention, the novel lytic reagent system comprises an aqueous solution containing a differentiation effective amount of lytic reagent selected from the group consisting of formic acid, acetic acid and their respective mixtures. In these mixtures, the formic acid will

preferably comprise the major functional component with the acetic acid being present in only minor amounts. The phrase "differentiation effective amount" is used throughout this disclosure as indicative of a concentration of lytic reagent which is not only effective for lysing red blood cells, but also effects subtle changes in the leukocyte cell fraction to facilitate the subsequent isolation, identification and/or analysis of this leukocyte cellular fraction; including the ability of instrumentation to perform differential analysis and identification of at least five (5) sub-populations of leukocytes. The preferred range of concentration of formic acid in the lytic reagent is in the range of from about 0.10 to about 0.25% (v/v). The concentration of such preferred lytic reagent which has been determined as satisfying the foregoing criteria is from about .009 to about 0.020 milliliters of formic acid per milliliter of whole blood. This subtle modification of the leukocyte fraction by the lytic reagent is achieved while preserving the immunochemical response of the surface markers of each of the five (5) cell sub-populations of leukocytes. As stated previously, it is the primary objective in this treatment of the whole blood sample with the lytic reagent, that such reagent effectively accomplishes stromatolysis of the erythrocyte cell fraction while preserving the leukocyte fraction in its essentially native state.

In one of the preferred embodiments of this invention, the reagent system can contain saponin in addition to the lytic reagent. The term "saponin" is intended as referring to commercial grade quillaja saponin powder. The addition of saponin to the reagent system is optional and generally only appropriate where the clinician is monitoring certain parameters of the leukocyte sub-populations other than by photooptical or immunochemical techniques. The addition of appropriate quantities of saponin to the lytic reagent system is effective in its ability to reduce the size of red cell fragments so as to prevent

their interference in determination of certain leukocyte parameters by measurement of electrical opacity and/or Coulter volume utilizing the techniques described in Coulter U.S. Patents 2,656,508 and 3,502,974 (which are hereby incorporated by reference in their entirety). The preferred range of concentration of saponin which has been determined as effective for reducing red cell fragments is from about 0.006 to about 0.012 grams per milliliter of whole blood. In brief, the technique involves the measurement of cell volume utilizing radio frequency current (RF) and DC field excitation. By generating a particle sensing field with both a low frequency or direct current (DC) and radio frequency (RF) current excitations, two or more interrelated output signals can be derived from passage of a single particle (i.e. leukocyte) through an electric field. The value derived from this output signal is termed "relative opacity", which is unique for each sub-population of leukocyte. The addition of saponin to the lytic reagent reduces the size of the red blood fragments to a point where they will not interfere with or themselves cause, the derivation of an output signal indicative of a species of leukocyte.

Where cell differentiation is based upon light scatter measurements, (utilizing the techniques described in Fulwyler U.S. Patent 3,989,381 and/or Auer, et al U.S. Patent 4,038,556 - which are hereby incorporated by reference in their entirety), the red cell fragments do not interfere or adversely effect the photometric differentiation of the various sub-populations of leukocytes. Thus, the addition of saponin to the lytic reagent system is unnecessary where such differentiation is based upon photometric analysis.

The amount of time of exposure of the blood sample to the lytic reagent system is critical to the differentiation method of this invention. This exposure period, as illustrated in the Examples

disclosed hereinafter, should not exceed ten (10) seconds, and most preferably, requires only about six (6) seconds or less. Both of these exposure times are specified for room temperature (~18-28°C). In each instance, the action of the lytic reagent is quenched by simple addition of appropriate concentrations of salts to the sample to return the cells to their native physiological environment. The quench effectively retards further activity of the lytic reagent upon the leukocytes without need for the addition of fixatives. The quench for the lytic reagent is an essential complement to the lytic reagent system where subsequent analysis of the leukocyte fraction requires retarding the activity of the lytic reagent. The leukocytes are stabilized by this quench by controlling the pH within a fairly narrow range (pH ~6.00 to 7.25) and osmolality (~300 to 330 milliosmoles). The quench can also be formulated to match the conductivity of a chosen "sheath" fluid which is utilized in a focused flow aperture analysis system. The composition and volume of the quench is adjusted to provide optimal separation of the five (5) major leukocyte subclasses when analyzed in accordance with the techniques (RF frequency current in combination with DC field excitation) described in Coulter, et al U.S. Patent 3,502,974 (previously incorporated by reference). The leukocyte fraction of the sample, treated in the above manner, can be readily differentiated to at least five (5) sub-populations by a hematology analyzer capable of multiple parameter particle (cell) measurements; and, by immunochemical interaction with antiserum (i.e. antibodies, binding proteins, etc.) that are specific for one or more surface markers on the cells of each such cell sub-population.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1, 2 and 3 are scattergrams representing the white cell differential analysis of the blood sample of Example II; the X-axis of each such Fig. being different from one another.

Fig. 4 is a scattergram representing the white cell differential analysis of the blood sample of Example I.

Fig. 5 is a scattergram representing the white cell differential analysis of the blood sample of Example V.

Fig. 6 is a scattergram representing the white cell differential analysis of the blood sample of Example VI.

DESCRIPTION OF THE INVENTION INCLUDING PREFERRED EMBODIMENTS

The lytic reagent system of this invention comprises an aqueous solution containing surprisingly low concentrations of the lytic reagent (preferably less than 1.0% by volume). The lytic reagent of the reagent system of this invention can generally be described as a water soluble, acidic compound which at least partially dissociates in aqueous media. As noted above in the Summary of the Invention, a number of interrelated factors are believed to be essential for such compounds to perform within the various environments contemplated for this invention. These factors include pH, osmolality and counterion compatibility. The

acidification of the sample is necessary to effect the desired lysis of the erythrocytes. Where, however, the pH of this sample departs from the preferred range of from about 2.6 to about 4.0, the ability to differentiate the leukocyte fraction into five (5) distinct subpopulations is apparently compromised.

This ability to effectively differentiate the leukocyte population into its various subpopulations is also hindered where the osmolality of the lysed sample is not maintained below about 100 mOs. Accordingly, where either pH or osmolality of the lysed sample is not maintained within the foregoing parameters, the ability to effectively differentiate between the various subpopulations of leukocytes is significantly impaired.

The counterion of the dissociated acidic compound has, in certain instances, been shown to effect the degree to which differentiation of the leukocyte fraction can be effected. For example, phosphoric acid, even at concentrations which produce the appropriate pH and osmolality, is not (as) effective in the differentiation of this leukocyte fraction into its various subpopulations. It is hypothesized that the counterion of this acid (phosphate) interacts in some unknown manner or fails to interact with the leukocyte fraction, and thereby does not afford the extent of differentiation afforded by other water soluble, acidic compounds.

There may also be other factors and mechanisms which are inherent in the action of the lytic reagents upon the sample which account for the surprising and unexpected results of this invention. As of the present, only the above three (3) enumerated variables have been identified; and it is not intended to imply that there may not be others or that this invention is dependant upon only the above identified variables for its effectiveness.

The lytic reagent of this invention is preferably an aqueous solution of formic acid, acetic acid or mixtures of formic and acetic acid in which formic acid is the predominant functional component. This aqueous solution is prepared by simple addition of the lytic reagent to deionized water. The amount of lytic reagent added to this diluent is sufficient to prepare a solution containing from about 0.05 to about 0.5% (v/v) solution. In the preferred embodiments of this invention, the concentration of lytic reagent will range from about 0.1 to about 0.25% (v/v).

Where the lytic reagent comprises a mixture containing both formic and acetic acid, the acetic acid is preferably only present as a partial replacement for a definitive amount of formic acid and then only at a concentration in the range of from about 0.05 to 0.10% (v/v).

The lytic reagent system also optionally can contain very small amounts of saponin (preferably at least about 0.05 up to about 0.2 weight percent) in addition to the lytic reagent. As is fully appreciate, the effectiveness of saponin, as a lytic reagent, is highly concentration dependent. If saponin is used at low concentrations, it is generally ineffective for this purpose. Were saponin to be used as a lytic reagent, it would have to be present in concentrations of at least 2.0 weight percent, or more, to effect essentially complete stromatolysis of the red blood cells. Unfortunately, the saponin, where present at a lytic effective concentration, can also cause lysis of white blood cells. Thus, any changes induced in the white blood cell population utilizing saponin as the lytic agent, and their subsequent differentiation by automated instrumentation must be based upon whatever discernible and distinctive characteristic of their respective nuclei. Its presence in the lytic reagent system of this invention

is, however, desirable where the clinician is concerned with so-called "ghosts" (intact red cell membranes) interfering with differential measurements based upon distinctions in physical and/or electrical properties. As noted previously, saponin would not be a necessary addition to the lytic reagent system if the differential measurements where made by purely photooptical instrumentation and/or by immunochemical analysis.

The lytic reagent system can also contain other traditional additives, to the extent their presence is not otherwise incompatible with the primary functional components of the system (i.e. anti-microbial preservatives, such as sodium omadine.

The lytic reagent system of this invention can be combined with a whole blood sample by simple manual or automated addition, the lytic reagent and sample allowed to briefly interact, and the action of the lytic reagent substantially retarded by addition of a suitable quench. The quench, to be effective in this environment, must, thus, be capable of retarding the lytic activity of the lytic reagent immediately upon its addition to the aqueous mixture contained in the blood sample and the lytic reagent. The precise formulation of the quench can vary, depending upon the composition of the lytic reagent system and the sheath fluid used in a focused flow aperture analysis system. The quench is typically an aqueous solution containing soluble salts which are both effective to substantially retard and/or substantially neutralize the lytic activity of the lytic reagent and restore the ionic balance to the sample. This restoration of the ionic balance will extend the longevity of the surviving cells and permit subsequent analysis on equipment which requires that the sample be electrically conductive (contain electrolytes), as for example with a Coulter Counter® whole blood analyzer.

A quench which is suitable for use in conjunction with a lytic reagent composition of this invention can, and usually will, contain any combination of at least two of the following four ingredients: sodium chloride, sodium sulfate, sodium bicarbonate, sodium carbonate; and, in addition, sodium azide as a preservative. The effectiveness of the quench upon the lytic reagent in the context of this invention is determined by its ability to rapidly reduce the lytic activity of the carboxylic and non-volatile mineral acids selected for use in the lytic reagent system. As noted above, the method and equipment utilized in the differentiation of the leukocyte sub-population can also place certain requirements (i.e. conductivity, pH, etc.) upon the precise formulation of the quench. More specifically, when such differentiation is performed in a focused flow aperture analysis system, the composition and volume of quench can be critical to optimal separation (differentiation) of the five (5) leukocyte subclasses from one another. In this type of analysis system, the ionic balance of the quench must also be adjusted to obtain a satisfactory conductivity match of the lysed blood sample to the sheath fluid. In the preferred quench formulation, the major ionic species and their relative ratio in the lysed-quenched blood sample should be essentially the same as the major ionic species and their relative ratio in the sheath fluid. The relative concentration of the functional components of the quench, which is to be used in conjunction with the lytic reagent system of this invention, will range from about 1 to about 3% (w/v) sodium chloride, about 0.25 to about 0.8% (w/v) sodium carbonate or bicarbonate, and about 2 to about 4% (w/v) sodium sulfate. The precise relative quantities of ingredients of the optimum quench are generally determined empirically; the objectives of such adjustment being to attain the pH of the lysed blood sample within the range of from about 6.0 to about 7.5, and a final osmolality of the stabilized lysed blood sample in the range of

from about 300 to about 330 mOs. It has been previously observed, in a focused flow aperture system, that optimal clustering of the leukocyte subclasses is achieved by adjustment in the osmolality of the final blood sample to about 310 mOs. The essentially complete neutralization of the acidity of the lysed sample with an alkaline quench can be critical to a focused flow aperture analysis system. Components of the sample (i.e. fibrin and platelets) are pH sensitive and can form aggregates under acidic conditions which can potentially interfere with differential analysis (i.e. noise) or physically obstruct the aperture of a focused flow aperture system.

It is not contemplated, nor intended, that the quench also necessarily inhibit or neutralize the saponin (when present). The reasons for this are quite simple, in that the saponin, at the concentrations contemplated (~0.2 weight percent), is relatively ineffective as a lytic reagent. If the quench were to also inhibit the saponin activity upon its addition to the sample, adequate clarification of the sample (destruction of intact red blood cell membranes) would not take place. Thus, it is anticipated that the activity of the saponin continue of from about 5 to 15 seconds subsequent to quenching of the activity of the lytic reagent. It may, under some circumstances, be appropriate to provide an independent quenching agent for the saponin; however, at the concentrations presently contemplated, (less than 0.1%) none would appear necessary to achieve or further the objectives of this invention. The adequacy of the quenching agent is, of course, based upon certain assumptions relating to sensitivity of cells of sample (analytes of interest) to the lytic reagent and the period of contact of the lytic reagent and these cellular analytes prior to analysis. As noted above, the quench retards the lytic activity, it does not totally eliminate its effect upon the leukocyte fraction of the sample. Thus, if a substantial period of time is to elapse between addition of quench and analysis of the sample, it may be

desirable to fix the leukocyte fraction to preserve the characteristic size and shape of the cellular analytes of interest.

In the preferred embodiments of this invention, the duration of effective contact of the lytic reagent and the blood sample (from the time the two are combined, to the time when the quench is added), must be less than ten (10) seconds, and most preferably six seconds or less. The interval of reactive contact of the lytic reagent and blood sample as specified above, presumes such reactive contact occurs at room temperature (~18-28°C). Of course, if the temperature is in excess of this level, the period of reactive contact would be somewhat less and vary inversely; and, if the temperature is lower than this level, the period of reactive contact would be somewhat longer. It is both critical and essential to the successful performance of the differential method of this invention, that the kinetics of the interaction of the lytic reagent upon both the sacrificial cell population (red blood cells) and the desirable cell fraction (leukocytes) be controlled carefully and precisely. The mechanism by which the lytic reagent reacts with both cell fractions is not known with precision, only the manifest effect of such interaction. It is, thus, beyond the scope of this discussion to speculate how these improvements in differential analysis are attained and, thus, no attempt is made herein to explain or later claim such mechanism.

By limiting the exposure of both these cell fractions to the lytic reagent, stromatolysis of the erythrocytes is effectively and efficiently accomplished, while additional subtle changes are induced in the leukocytes by the quench to enable their effective differentiation. Both of these events occur essentially concurrently, while preserving the native immunochemical reactivity of the differentiated leukocyte fraction.

As noted above, the duration of contact of the lytic reagent with the blood is sufficient to effect a selective destructive response of the sacrificial cell fraction, while at the same time effecting a differential response in the leukocyte fraction; such changes unexpectedly permitting the physical differentiation of at least five (5) cell sub-populations of leukocytes from one another. The lytic reagent, in sharp contrast to the more traditional types of lyse (i.e. saponin, quaternary ammonium salts), does not detrimentally alter the native immunochemical response of the surface markers of each of the cells within the leukocyte sub-populations. This quality is believed to be unique to the lytic reagents of this invention.

Blood samples which have been exposed to the above lytic reagent system can be subjected to differential measurement on instrumentation designed for this purpose. Such differentials can be performed on a device utilizing technology of the type described in U.S. Patents 3,549,994 (which is hereby incorporated by reference in its entirety), 3,502,974 and 3,989,381. Features of the inventions described in the above referenced patents can be embodied into commercially practical instrumentation. In brief, the blood sample is initially treated by mixing with the lytic reagent system, utilizing automated pipetting equipment. The lytic action of the lytic reagent on the sacrificial cell population (red blood cells), must be both rapid and effective. A quench then is added by similar pipetting means to substantially retard the activity of the lytic reagent upon the surviving cell fraction (leukocyte). The sample containing the leukocyte fraction then is subjected to counting of each of the individual cell sub-populations, and/or histograms and/or scattergrams generated from the data collected in this fashion.

The principle involved in performance of Coulter volume measurements is well known to those skilled in the art as the "Coulter Principle." In brief, the operation of instrumentation utilizing the Coulter Principle involves the measurement of change in the impedance caused by the passage of individual cells through a sensor designed to detect a voltage drop caused by the presence of the cell. The instrumentation utilizing this principle comprises two fluid vessels or chambers, each containing a conductive electrolyte solution. At least two electrodes having opposite polarity are immersed in the electrolyte solution, with each fluid compartment having one of the electrodes disposed therein. A sample of the electrolyte solution, having the blood cells suspended therein, is passed through a constricted fluid path, or orifice, interposed between the two fluid compartments. Although the constricted pathway can take different forms, in each device such path defines a sensing zone wherein the presence or absence of a particle gives rise to a detectable change in electrical characteristics of the path. For example, relatively poorly conductive blood cells passing through this path, displace a volume of electrolyte solution equal to the cell volume, causing a voltage drop by increasing the path impedance. The resistance pulses defined by the drops in voltage are used for particle counting and particle volume determination. The Coulter principle is more fully described in U.S. Patent 2,656,508. This technique for sensing and identifying specific cell populations can be enhanced by a combination of Coulter principle measurements with radio frequency excitation of the cells within the sensing zone. In brief, this radio frequency enhancement operates upon the principle that a particle moving through the sensing zone of a hematology analyzer will cause a phase shift in radio frequency (RF) energy within the sensing zone. This shift in phase can be correlated with physical and compositional characteristics of a cell population. This technique for RF differentiation of cells is more fully described in U.S. Patent 3,502,974. White cell differentiation also can be achieved utilizing optical measurement

principles of flow cytometry as described in U.S. Patent 3,380,584 (which is hereby incorporated by reference in its entirety).

The lytic reagent system of this invention is effective to induce subtle changes in the leukocyte cell fraction to enhance the differentiation of five (5) distinct sub-population of leukocytes on automated cell counting equipment, see for example, U.S. 4,412,004 (to Ornstein, et al - which is hereby incorporated by reference in its entirety). These five (5) distinct sub-populations, as noted previously, include: lymphocytes, monocytes and three species of granulocytes (eosinophils, basophils and neutrophils). Each of these sub-populations of leukocytes have distinctive surface markers. In certain disease states, the surface markers on one or more of these sub-populations will provide a unique immunochemical response and, thus, make diagnosis or confirmation of disease possible at an early stage of its development. The detection of these distinctive surface markers on one or more of these sub-populations of leukocytes will, of course, be dependent upon the ability to effectively physically isolate these cells having characteristic disease state surface markers from the non-effected cells; and, the relative concentration of the effected cells within the sample being analyzed.

The initial lytic conditions contemplated by this invention permit an extension in the longevity of the cell populations which survive and which are to be differentiated from one another. For the most part, it is anticipated that cell longevity of at least seventy-two (72) hours would be appropriate for certain types of immunochemical analysis. In certain applications of this differential method, it may be both necessary and appropriate to maintain one or more of these sub-populations <u>in vitro</u> for several hours, or possibly even several days. In order to achieve

such extended stability, it is advisable to physically separate the leukocytes from the fluid fraction containing the lytic reagent/quench mixture and, thereafter, resuspended such cells in a physiological medium.

The following examples are provided as illustrative of the unique advantages of the lytic reagent system of this invention. The equipment and techniques utilized in the preparation and evaluation of this lytic reagent system are standard or as hereinbefore described. Parts and percentages appearing in such Examples are by weight unless otherwise stipulated.

EXAMPLE I

The lytic reagent system of this invention was prepared from reagent grade chemicals. A 0.12% (v/v) formic acid solution was initially prepared by combining 1.3 ml of 90% formic acid and 998 ml deionized water. A 50 µL whole blood sample (K_3 EDTA) and 800 microliters of 0.12% formic acid solution are gently mixed by swirling the two together for five (5) seconds at room temperature (~ 20°C). The lytic action of the formic acid arrested after about 5 seconds, by the addition of 400 µl of a quench solution containing 0.55% sodium bicarbonate, 3.0% sodium chloride and 0.01% sodium azide. The sample was adequately quenched and ready for differential analysis by flow cytometry techniques within about 5 to 10 seconds subsequent to the addition of the quench. The equipment used in such differential analysis was equipped with a helium/neon laser and silicon diode detectors for measurement of light scatter. The leukocytes are observed and their individual parameters determined by optical parameter analysis, i.e. measurement of light extinction (zero angle scatter), and any one of several angular ranges of light

scatter. The scattergram of the sample generated in the above manner is illustrated as Figure 4. Five (5) distinct sub-populations of leukocytes are identified and quantified in this scattergram.

EXAMPLE II

The procedures of Example I are repeated, except for the addition of 0.05% (w/v) saponin powder to the aqueous solution containing 0.12% formic acid. The whole blood sample (50 microliters) is then combined (as previously described) with 600 microliters lytic reagent. The lytic reagent is quenched after 6 seconds by addition of 265 microliters of an aqueous solution containing 0.60% sodium carbonate, and 3.00% sodium chloride.

The addition of saponin to the lytic composition effectively eliminates interference of the red cell debris from Coulter volume measurements. The effectiveness of the saponin is highly temperature dependent. The essentially total elimination of interference from red cell debris requires an additional 10 seconds (at room temperature ~18 to 28°C) subsequent to completion of lysing the red cell fraction of the sample. In the event the sample is maintained at a lower temperature (below 18°C), a somewhat longer period will be required to effectively eliminate interference from the red cell debris by the saponin.

The sample was ready for differential analysis within about 10 to 20 seconds subsequent to the addition of the quench. The sample was subjected to photo-optical measurement as described in Example I. Leukocytes in the sample were also observed by measurement of DC and RF volumes using an ISOTON® II sheath

fluid and the resultant scattergram is illustrated in Fig. 1. Four distinct sub-populations of leukocytes were identified and quantified in the simultaneously obtained light scatter vs. DC scattergram of Fig. 2. A fifth sub-population of leukocyte (basophils) is isolated by generation of a "gated" secondary scattergram. This basophil population is depicted in the scattergram illustrated in Fig. 3.

EXAMPLE III

The procedures of Example II are repeated, utilizing the same formic acid/saponin reagent composition. The lytic activity of the formic acid is arrested with a quench containing 3.13% (w/v) sodium sulfate (anhydrous), 1.45% (w/v) sodium chloride and 0.60% (w/v) sodium carbonate (anhydrous). The sample was analyzed by electro-optical techniques in the manner described in Example II and the scattergram results were similar to that of Figs. 1,2 and 3. The sheath fluid was, however, changed to ISOTON® III diluent.

EXAMPLE IV

The procedures of Example III were repeated, except for use of a concentrated lysed-quenched blood sample for more rapid data acquisition. The lytic reagent comprises 0.15% (v/v) formic acid and contains 0.10% (w/v) saponin powder. The lytic activity of the formic acid was arrested by a quench containing 2.67% (w/v) sodium sulfate (anhydrous), 1.24% (w/v) sodium chloride and 0.56% (w/v) sodium carbonate (anhydrous).

This differential analysis was performed by addition of 50 microliters whole blood to a glass culture tube containing 300 microliters lyse reagent. The sample and lyse are mixed by swirling the contents of the tube for approximately 6 seconds and the lytic activity of the formic acid arrested by addition of 165 microliters of the above quench. The sample was then subjected to electro-optical measurement in the manner described in Example II and the resultant differential analysis was comparable to that of Example II, (as illustrated in Figs. 1,2 and 3).

EXAMPLE V

The procedures of Example I were repeated, except for the substitution of 0.1% (v/v) acetic acid for the formic acid in the lytic reagent system. The lytic activity of the reagent was arrested, after approximately 5 seconds, with a quench comprising 0.25% sodium bicarbonate in 2.0% sodium chloride solution. The sample was adequately quenched and ready for differential analysis by flow cytometry techniques within about 5 to 10 seconds subsequent to the addition of the quench. The equipment and analytical techniques used in such analysis were essentially the same as in Example I. The differential analysis performed on this sample is illustrated in the scattergram of Fig. 5.

EXAMPLE VI

The procedures of Example V were repeated except for extending the period of contact of the sample and the lytic reagent from 5 to 7 seconds prior to the addition of the quench. The quench was also modified slightly by increasing the concentration of sodium

bicarbonate from 0.25% to 0.375% in the 2.0% sodium chloride solution. The results of this differential analysis of the leukocyte population are illustrated in the scattergram of Fig. 6.

EXAMPLE VII

The procedures of Example I are repeated, except for the separation and identification of the various sub-populations of leukocyte by immunochemical techniques. Once the action of the lytic reagent is quenched, the sample is diluted with an isotonic buffer and sequentially slurried with a series of magnetic particles, each of which having been pre-treated with a different antiserum specific for adsorption of only one species of leukocytes. The adsorbed cells can be separated sequentially from the sample using the conventional magnetic particle separation techniques described in the previously incorporated references. The separated particles can then undergo additional screening for surface markers which are indicative of one or more disease states.

EXAMPLE VIII

The procedures of Example VII are repeated, except that the cells are separated from the magnetic particles and cultured with an immortal cell line in accordance with the procedures of Kohler and Milstein, Nature, Vol. 256, 495-497 (1975). The clones produced in this fashion are screened for the production of antiserum specific for the surface marker of interest.

EXAMPLE IX

The procedures of Example V are repeated, except for the substitution of 0.1% (v/v) citric acid for the acetic acid in the lytic reagent system. This substitution achieved results comparable to that attained in Example V.

EXAMPLE X

The procedures of Example V are repeated, except for the substitution of 0.1% (v/v) succinic acid for the acetic acid in the lytic reagent system. This substitution achieved results comparable to that attained in Example V.

EXAMPLE XI

The procedures of Example V are repeated, except for the substitution of 0.1% (v/v) lactic acid for the acetic acid in the lytic reagent system. This substitution achieved results comparable to that attained in Example V.

EXAMPLE XII

The procedures of Example V are repeated, except for the substitution of 0.05% (v/v) sulfuric acid for the acetic acid in the

lytic reagent system. This substitution achieved results comparable to that attained in Example V.

The foregoing Detailed Description and Examples are intended as illustrative of some of the preferred embodiments of the lytic reagents, reagent systems and methods of this invention. It is not the intent of the inventors that these specific embodiments of their invention be interpreted as indicative of the scope or breadth thereof, but rather simply supportive of the claims which are set forth hereinafter.

CLAIMS

1. A lytic reagent system for chemical treatment of a whole blood sample so as to effect the partitioning of said sample into two distinct fractions, an essentially intact leukocyte fraction and a lysed erythrocyte fraction, said system comprising a first aqueous solution having a lytic reagent comprising a water soluble compound which at least partially dissociates in aqueous media so as to generate free protons and counterions,

said system being characterized in that said first aqueous solution comprises a differentiation effective amount of a lytic reagent and that said system includes a second aqueous solution comprising a quench specific for said lytic reagent,

said differentiation effective amount of said lytic reagent, when added to the whole blood sample, effecting: (i) a decrease in the pH of the sample from its physiological level to a pH in the range of from about 2.6 to about 4.0 while maintaining the osmolality of the sample at less than about 100 mOs; (ii) rapid and essentially complete hemolysis of said erythrocyte fraction and; (iii) subtle changes in said leukocyte fraction to enhance the ability of instrumentation to perform differential analysis and identification of at least five (5) sub-populations of leukocytes, said subtle changes being effected while preserving said leukocyte fraction in its essentially native physiological and/or immunochemical state; and,

said second aqueous solution comprising an alkaline salt solution, which upon contact with said lytic reagent effectively and rapidly arrests the chemical action of said

lytic reagent upon the sample and restores the native physiological environment by stabilizing the lysed sample at a pH in the range of from about 6 to about 7.5 and an osmolality in the range from about 300 to about 330 mOs, so as to permit the analysis of the leukocyte fraction in its native or near native condition.

2. The lytic reagent system of claim 1, characterized wherein said lytic reagent has the following formula

R - COOH

wherein R is H; an aliphatic hydrocarbon radical having from 1-3 carbon atoms; a carbonyl substituted aliphatic hydrocarbon radical having from 1-3 carbon atoms; a hydroxy substituted aliphatic hydrocarbon radical having from 1-3 carbon atoms; or an aliphatic hydrocarbon radical having from 1-3 carbon atoms and multiple carbonyl and/or hydroxy substituents.

3. The lytic reagent system of claims 1 or 2, characterized wherein said lytic reagent is selected from the group consisting of formic acid; acetic acid; citric acid; succinic acid; lactic acid; or, their respective mixtures.

4. The lytic reagent system of claims 1 or 2, characterized wherein said concentration of lytic reagent in said first aqueous solution, can range from about 0.01 to about 1.0% (v/v).

5. The lytic reagent system of claims 1 or 2, characterized wherein said concentration of lytic reagent in said first aqueous solution can range from about 0.05 to about 0.5% (v/v).

6. The lytic reagent system of any one of claims 1, 2, 3, 4 or 5, characterized wherein said first aqueous solution is further characterized as having an additional active component, said additional active component comprising saponin and being present at an erythrocyte stroma clarification effective amount in the range of from about 0.05 to about 0.20% (w/v).

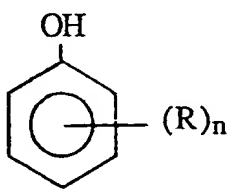
7. The lytic reagent system of claim 1, characterized wherein said lytic reagent has the following formula:

$$R\text{-}SO_3H$$

wherein R is hydroxy, an aliphatic hydrocarbon radical of 1-3 carbon atoms, or aryl.

8. The lytic reagent system of claim 7, characterized wherein said lytic reagent is selected from the group consisting of sulfuric acid; methanesulfonic acid; ethanesulfonic acid; benzenesulfonic acid; p-toluenesulfonic; m-nitrobenzenesulfonic acid; and, their respective mixtures.

9. The lytic reagent of claim 1, characterized wherein said lytic reagent has the following formula:



wherein R is an electron withdrawing group such as halogen, cyano, nitro, or any combination of said electron withdrawing group; and
n is 1-3.

10. The lytic reagent system of claim 9, characterized wherein said lytic reagent selected from the group consisting of para-nitrophenol; meta-nitrophenol; ortho-nitrophenol; 2,4-dinitrophenol; para-chlorophenol; para-cyanophenol; 1-chloro-2,4-dinitrophenol; and, their respective mixtures.

11. A method for partitioning a whole blood sample into two distinct fractions, an essentially intact leukocyte fraction and a lysed erythrocyte fraction, said method being characterized by the following steps performed in the following order:

(a) providing a lytic reagent system having:

(i) a first aqueous solution comprising a diluent and a lytic reagent comprising a water soluble compound which at least partially dissociates in aqueous media so as to generate free protons and counterions, said first aqueous solution being characterized as containing a differentiation effective amount of said lytic reagent,

said differentiation effective amount of said compound, when added to the whole blood sample, effecting: (i) a decrease in the pH of the sample from its physiological level to a pH in the range of from about 2.6 to about 4.0 while maintaining the osmolality of the sample at less than about 100 mOs; (ii) rapid and essentially complete

hemolysis of the red blood cell fraction and; (iii) subtle changes in the leukocyte cell fraction to enhance the ability of instrumentation to perform differential analysis and identification of at least five (5) sub-populations of leukocytes, said subtle changes being effected while preserving the leukocyte sub-populations in their essentially native physiological and/or immunochemical state; and,

(ii) a second aqueous solution consisting essentially of an alkaline salt solution of a quenching reagent, which upon contact with the lytic reagent effectively and rapidly arrests the chemical action of said lytic reagent upon the sample and restores the native physiological environment by stabilizing the lysed sample at a pH in the range of from about 6 to about 7.5 and an osmolality in the range from about 300 to about 330 mOs, so as to permit the analysis of the leukocyte fraction in its native or near native condition;

(b) contacting said whole blood sample with said differentiation effective amount of said lytic reagent system;

(c) allowing said lytic reagent and said sample to interact for an interval not to exceed ten seconds at a temperature in the range of from about 18 to 28°C; and,

(d) retarding the interaction of said lytic reagent upon said leukocyte fraction of the sample by addition of a quenching reagent, said quenching agent being present in sufficient concentration to essentially immediately retard the lytic activity of the lytic reagent and restore the physiological environment of said leukocyte fraction within the sample.

12. The method of claim 11, characterized by utilizing the lytic reagent system of claim 2.

13. The method of claim 11, characterized by utilizing the lytic reagent system of claim 7.

14. The method of claim 11, characterized by utilizing the lytic reagent system of claim 9.

15. The method of any one of claims 11, 12, 13 or 14, characterized wherein the concentration of said lytic reagent in said first aqueous solution, can range from about 0.05 to about 0.5% (v/v).

16. The method of any one of claims 11, 12, 13, 14 or 15, characterized wherein said first aqueous solution is characterized as having an additional active component, said additional active component comprising saponin and being present at an erythrocyte stroma clarification effective amount in the range of from about 0.05 to about 0.20% (w/v).

17. A test kit for partitioning a whole blood sample into two fractions, an essentially intact leukocyte fraction and a lysed erythrocyte fraction, said test kit comprising a first aqueous solution which contains a lytic reagent which at least partially dissociates in aqueous media to generate free protons and counterions,

said test kit being characterized as having two distinct and complementary solutions, said first aqueous solution containing a lytic reagent which is effective, when present at a differentiation effective amount, to selectively lyse said erythrocyte fraction of said whole blood sample and a second complementary solution containing a quench, specific for said lytic reagent, which is effective when present at appropriate amounts, to retard the lytic activity of said lytic reagent upon the sample,

said differentiation effective amount of said lytic reagent, when added to the whole blood sample, effecting: (i) a decrease in the pH of the sample from its physiological level to a pH in the range of from about 2.6 to about 4.0 while maintaining the osmolality of the sample at less than about 100 mOs; (ii) rapid and essentially complete hemolysis of said erythrocyte fraction and; (iii) subtle changes in said leukocyte fraction to enhance the ability of instrumentation to perform differential analysis and identification of at least five (5) sub-populations of leukocytes, said subtle changes being effected while preserving said leukocyte fraction in its essentially native physiological and/or immunochemical state,

a quench consisting essentially of an alkaline aqueous salt solution which is effective, upon addition to the sample, to rapidly retard the lytic activity of said lytic reagent, while stabilizing the lysed sample at a pH in the range of from about 6 to about 7.5 and an osmolality in the range of from about 300 to 330 mOs.

18. The test kit of claim 17, characterized wherein said lytic reagent has the following formula:

R - COOH

wherein R is H; an aliphatic hydrocarbon radical having from 1-3 carbon atoms; a carbonyl substituted aliphatic hydrocarbon radical having from 1-3 carbon atoms; a hydroxy substituted aliphatic hydrocarbon radical having from 1-3 carbon atoms; or an aliphatic hydrocarbon radical having from 1-3 carbon atoms and multiple carbonyl and/or hydroxy substituents.

19. The test kit of claims 17 or 18, characterized wherein said lytic reagent is selected from the group consisting of formic acid; acetic acid; citric acid; succinic acid; lactic acid; or, their respective mixtures.

20. The test kit of claims 17 or 18, characterized wherein said concentration of lytic reagent in said first aqueous solution, can range from about 0.01 to about 1.0% (v/v).

21. The test kit of claims 17 or 18, characterized wherein said concentration of lytic reagent in said first aqueous solution is from about 0.05 to about 0.5% (v/v).

22. The test kit of any one of claims 17, 18, 19, 20 or 21, characterized wherein said first aqueous solution is further characterized as having an additional active component, said additional active component comprising saponin and being

present at an erythrocyte stroma clarification effective amount in the range of from about 0.05 to about 0.20% (w/v).

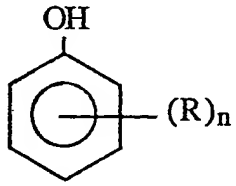
23. The test kit of claim 17, characterized wherein said lytic reagent has the following formula:

$$R\text{-}SO_3H$$

wherein R is hydroxy, an aliphatic hydrocarbon radical of 1-3 carbon atoms or aryl.

24. The test kit of claim 23, characterized wherein said lytic reagent is selected from the group consisting of sulfuric acid; methanesulfonic acid; ethanesulfonic acid; benzenesulfonic acid; p-toluenesulfonic; m-nitrobenzenesulfonic acid; and, their respective mixtures.

25. The test kit of claim 17, characterized wherein said lytic reagent has the following formula:



wherein R is an electron withdrawing group such as halogen, cyano, nitro or any combination of said electron withdrawing group; and n is 1-3.

26. The test kit of claim 25, characterized wherein said lytic reagent is selected from the group consisting of para-nitrophenol;

meta-nitrophenol; ortho-nitrophenol; 2,4-dinitrophenol; para-chlorophenol; para-cyanophenol; 1-chloro-2,4-dinitrophenol; and, their respective mixtures.

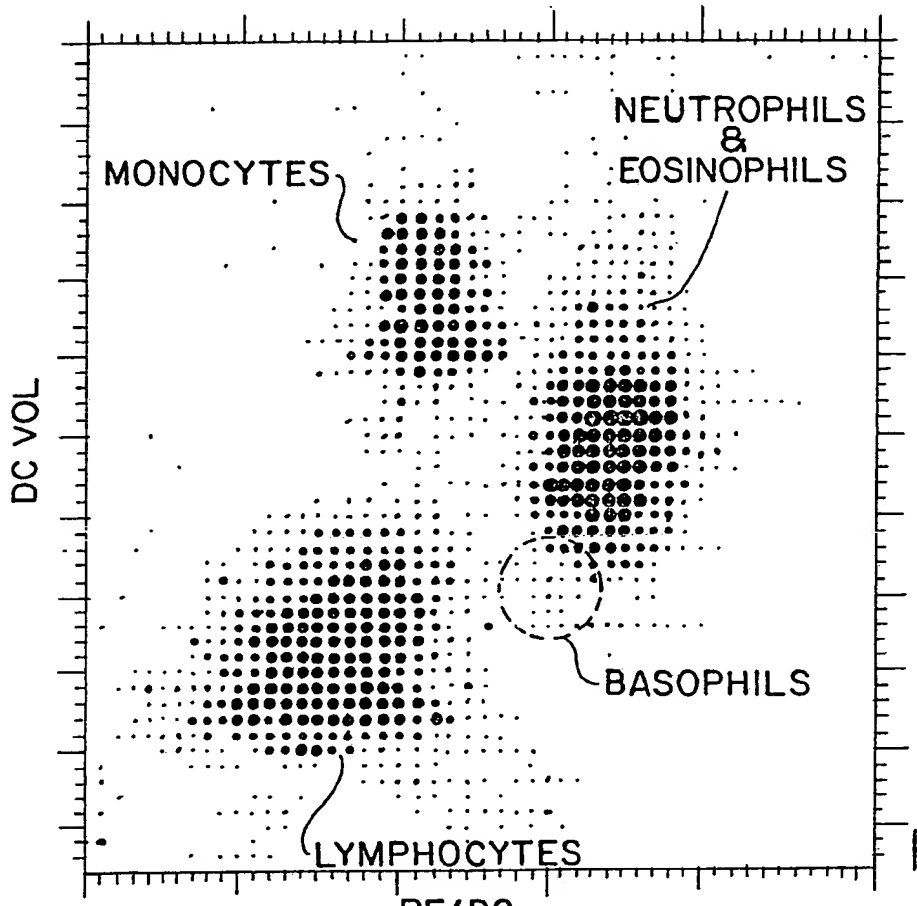

NEUTROPHILS
&
EOSINOPHILS
MONOCYTES
DC VOL
BASOPHILS
LYMPHOCYTES
RF/DC

FIG.1

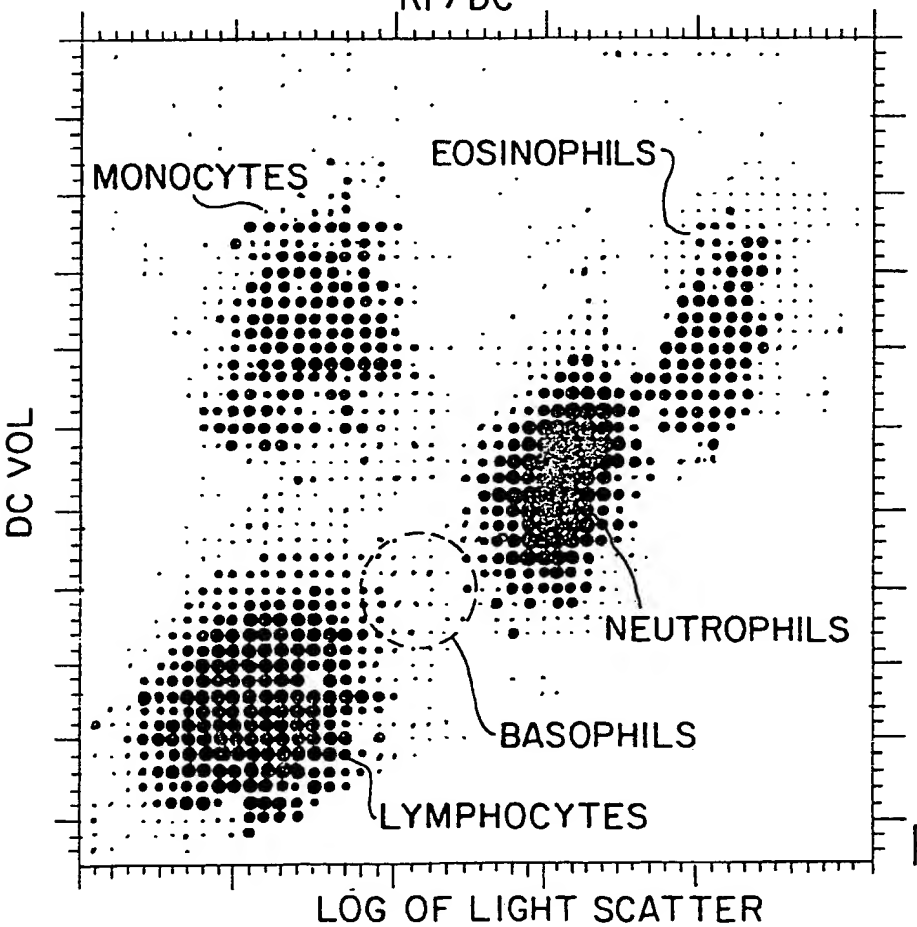

EOSINOPHILS
MONOCYTES
DC VOL
NEUTROPHILS
BASOPHILS
LYMPHOCYTES
LOG OF LIGHT SCATTER

FIG.2

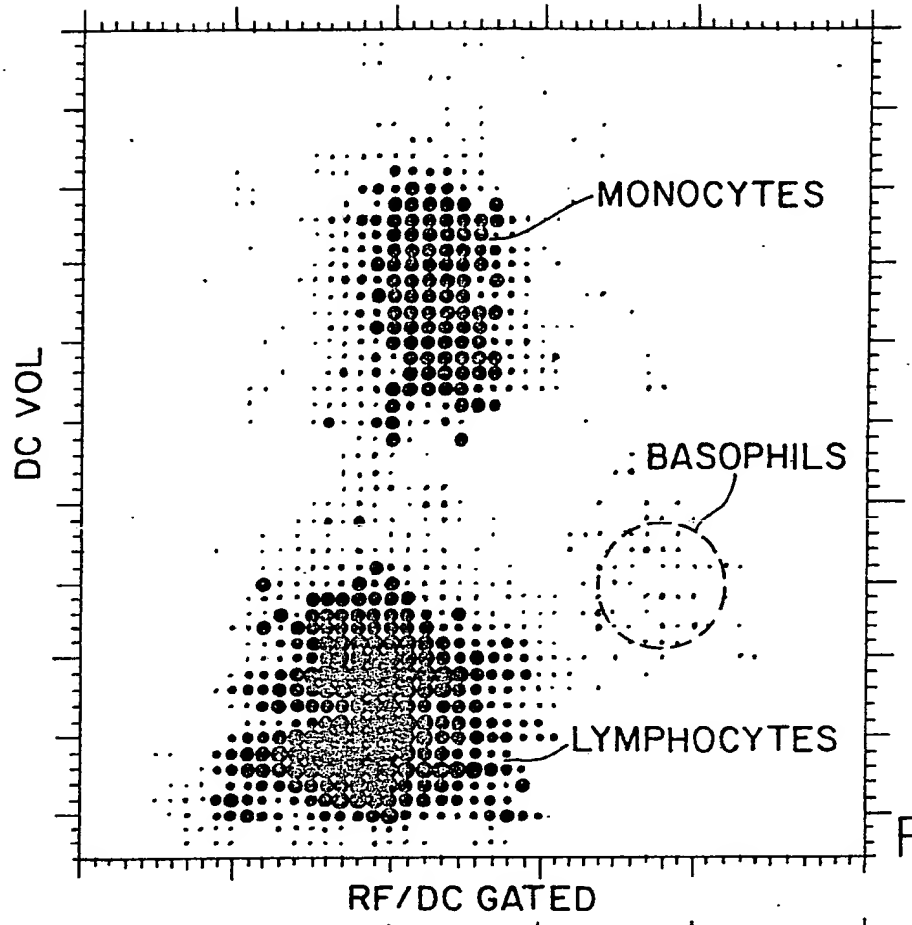

MONOCYTES
BASOPHILS
LYMPHOCYTES
DC VOL
RF/DC GATED

FIG.3

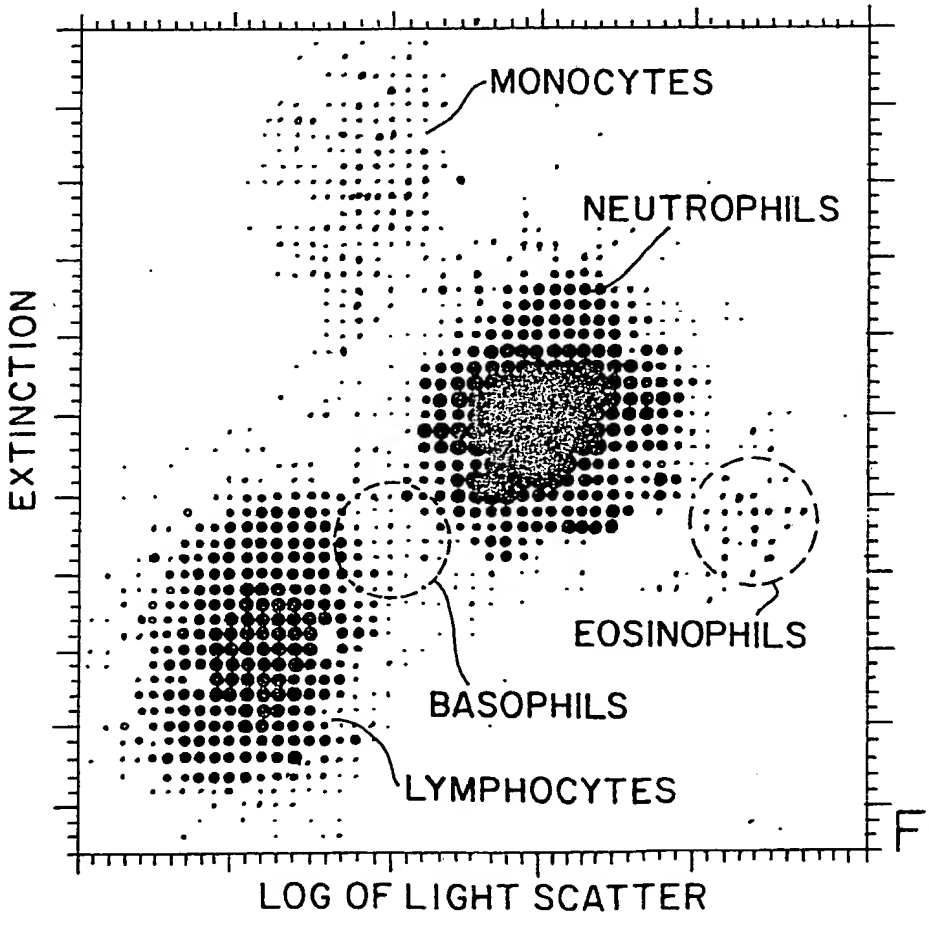

MONOCYTES
NEUTROPHILS
EOSINOPHILS
BASOPHILS
LYMPHOCYTES
EXTINCTION
LOG OF LIGHT SCATTER

FIG.4

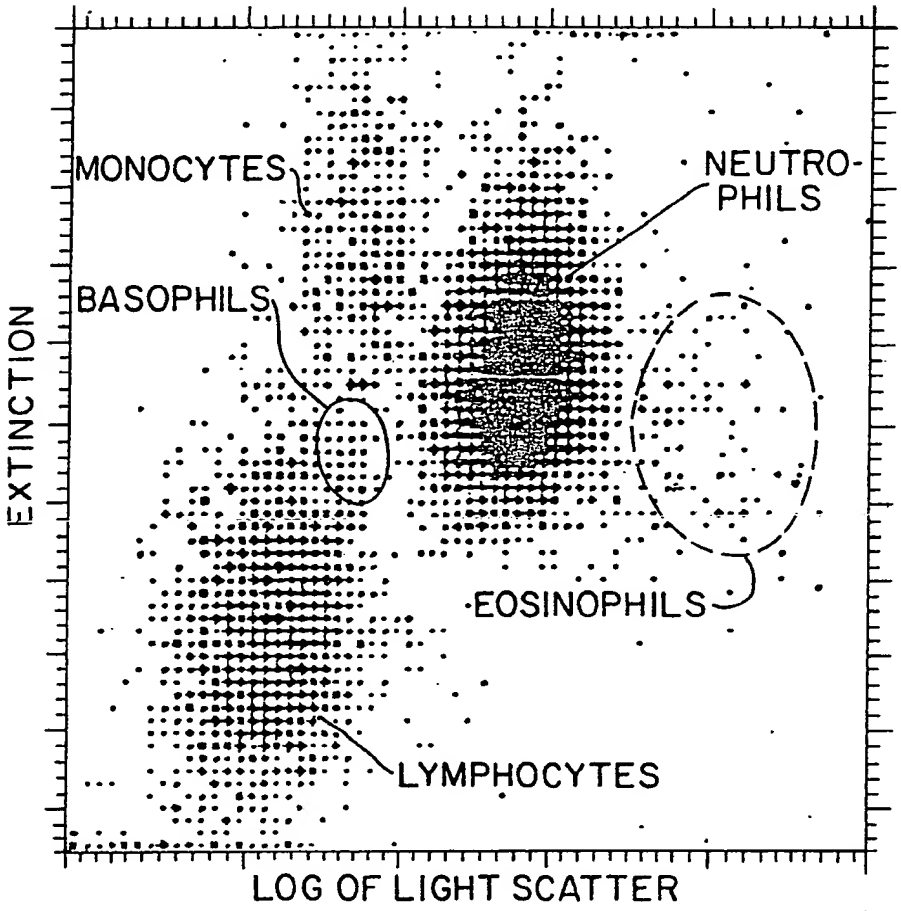

MONOCYTES
NEUTRO-
PHILS
BASOPHILS
EOSINOPHILS
LYMPHOCYTES
EXTINCTION
LOG OF LIGHT SCATTER

FIG.5

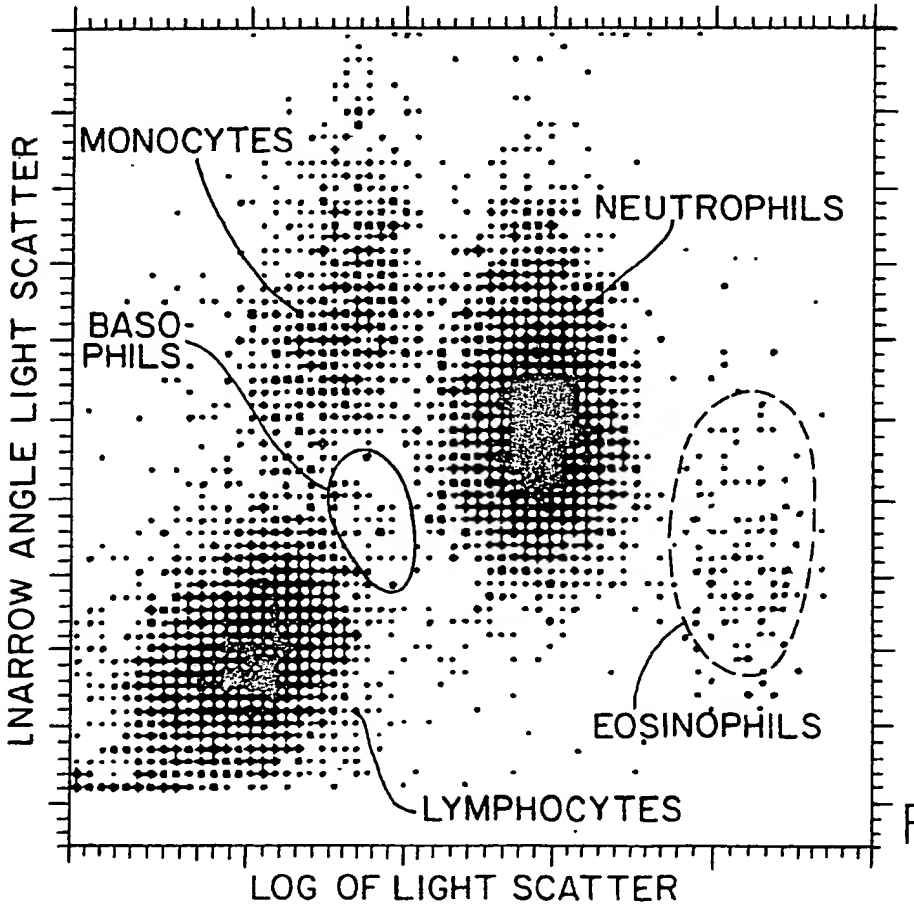

MONOCYTES
NEUTROPHILS
BASO-
PHILS
EOSINOPHILS
LYMPHOCYTES
LNARROW ANGLE LIGHT SCATTER
LOG OF LIGHT SCATTER

FIG.6

INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/00762

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): G01N 1/28, 33/50

US. CL.: 422/61; 436/17, 63, 175, 176

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
U.S.	422/61; 436/8, 10, 17 18, 63, 174, 175, 176, 179

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X Y	CA, A, 1,087,966 (BENVENISTE) 21 October 1980, see especially page 12, lines 20-29, page 18, lines 10-23, and page 34, lines 20-30.	1-5 6, 17-22
Y	US, A, 3,446,751 (WEICHSELBAUM) 27 May 1969, see the entire document.	6, 22
Y	US, A, 4,269,605 (DEAN et al) 26 May 1981 see especially column 6, lines 17-30.	17-22
A	US, A, 3,874,852 (HAMILL) 01 April 1975, see the entire document.	
A	US, A, 4,099,917 (KIM) 11 July 1978, see the entire document.	
A	US, A, 4,250,051 (ARMSTRONG) 10 February 1981, see the entire document.	
A	US, A, 4,286,963 (LEDIS et al) 01 September 1981, see the entire document.	

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09 June 1988	27 JUN 1988

International Searching Authority	Signature of Authorized Officer
ISA/US	Robert J. Hill, Jr.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
A	US, A, 4,324,685 (LOUDERBACK) 13 April 1982, see the entire document.	
A	US, A, 4,346,018 (CARTER et al) 24 August 1982, see the entire document.	
A	US, A, 4,436,821 (RYAN) 13 March 1984, see the entire document.	
A	US, A, 4,485,175 (LEDIS et al) 27 November 1984, see the entire document.	
A	JP, A, 54-68291 (MIKI ENGINEERING) 06 January 1979, see the abstract.	
A	J. MIALE, "Laboratory medicine-HEMATOLOGY", third edition, published 1967 by The C. V. Mosby Company (Saint Louis), see page 1122.	
A	Acta Paediatrica Scandinavica, volume 60, issued 1971, F. Schettini et al, "Acid Lysis of Red Blood Cells in Normal Children", see pages 17-21.	
A	Transfusion, volume 13, number 5, issued September-October 1973, A. da Costa et al, "Effects of Ethacrynic acid on Human Red Blood Cells", see pages 305-313.	
A	Zeitschrift fuer Immunologie-Forschungs-gemeinschaft, volume 155, issued 1978, U. Rother et al, "Deviated Lysis: Lysis of Unsensitized Cells by Complement. V. Generation of the Activity by Low pH or Low Ionic Strength", see pages 118-129.	